NATIONAL INSTITUTES OF HEALTH
NIH LIBRARY
NOV - 7 1997
BLDG 10, 10 CENTER DR.
BETHESDA, MD 20892-1150

RJ
85
N277
1996

CBMB

NICHD ANNUAL REPORT
BRANCH CHIEF'S SUMMARY
OCTOBER 1, 1995, TO SEPTEMBER 30, 1996
CELL BIOLOGY AND METABOLISM BRANCH

The Cell Biology and Metabolism Branch (CBMB) pursues studies in a variety of areas of cell and regulatory biology, including the structure and dynamics of the internal organization of eukaryotic cells, the biology and pathophysiology of metal metabolism, prokaryotic and eukaryotic gene regulation, the structural and biochemical basis of immune cell activation, and the biochemistry and cell biology of tumor suppressor genes. A wide array of techniques are brought to bear upon the scientific problems of interest to the investigators. In particular, CBMB has continued to expand its facilities and capabilities in microscopy with emphasis on electron-microscopy, including cryo-immunogold-microscopy, and light and fluorescence microscopy. A powerful confocal microscope has just been added to enhance the capacity to study intracellular fine structure and dynamics. A specially designed microscope has been built, coupled to a computer-based imaging system that has allowed the branch to explore more powerful ways of analyzing light, differential interference, and fluorescence microscopy. In addition, there are microinjection facilities both for *Xenopus* oocytes and a state-of-the-art mammalian cell microinjector. Facilities for the growth and study of the genetically tractable plant *Arabidopsis* are in operation. A new project to study mechanisms of gene transcription *in vivo* has been initiated. This work takes advantage of the power of *Drosophila* genetics. The Branch has continued its clinical interests in disease of metal metabolism and continues an extensive study aimed at a clinical and molecular characterization of iron overload in Africans and African Americans. This project, funded largely through the NIH Minority Health Program, includes a major field study in Zimbabwe and Swaziland aimed at identifying pedigrees in which iron overload is observed. The open structure of the laboratory, its shared facilities, seminars, group meetings, journal clubs, and an annual retreat, make the CBMB a highly interactive scientific environment.

The projects in the lab are overseen by seven senior investigators, Richard Klausner, Tracey Rouault, Lawrence Samelson, Juan Bonifacino, Gisela Storz, Jennifer Lippincott-Schwartz, and David Wassarman. The research of the laboratory can be broadly divided into five areas: (1) regulatory biology, (2) cell biology, (3) signal transduction, (4) tumor suppression, and (5) transcriptional regulation.

REGULATORY BIOLOGY

Three projects in the laboratory are aimed at understanding the mechanics and regulation of dealing with either nutritional or environmental stresses. Two of these projects are aimed at understanding the metabolism in eukaryotes of essential but toxic metals, in particular iron and copper. One of the projects uses prokaryotes to study the genetics and biochemistry of the response to oxidative stress.

REGULATION OF INTRACELLULAR IRON METABOLISM:

One group, co-directed by ***Tracey Rouault*** and ***Richard Klausner*** studies the regulation of intracellular iron metabolism in human cells. We have cloned and characterized through site-directed mutagenesis the two major regulatory proteins involved in sensing iron levels, IRP1 and IRP2.

A Sequence Unique to IRP2 is Required for Rapid Iron-Dependent Degradation

Binding studies have shown that IRP2 binds to consensus IREs with high affinity and specificity similar to IRP1. Whereas the RNA binding activity of IRP1 is regulated by the reversible assembly and disassembly of an iron-sulfur cluster in an otherwise stable protein, absolute levels of IRP2 are markedly decreased by iron treatment in several different cell types and the decrease in protein levels can be accounted for by an increase in the rate of degradation of IRP2. IRP1 and IRP2 are highly homologous proteins with an overall sequence identity of 58%. A major distinguishing feature between IRP1 and IRP2 is the presence of an insertion of 73 amino acids in domain 1 of IRP2. Cloning of the genomic fragment corresponding to this sequence has revealed that the 73 amino acids are encoded by a single exon. When the 73 amino acid insertion was excised by site directed mutagenesis from IRP2, levels of the IRP2 exon deletion mutant (IRP2-73) were no longer markedly decreased after treatment with iron. To assess whether the 73 amino acid IRP2-specific exon contained sufficient information to direct iron regulated degradation of IRP2, the sequence was cloned into IRP1 at a homologous position. When assayed in a pulse chase experiment in the presence of iron, the IRP1/IRP2 specific exon chimeric protein (IRP1+73) was much more rapidly degraded than wild-type IRP1 and the degradation rate was comparable to that of wild type IRP2 (t1/2 of approximately 3-6 h). Thus, the IRP2 specific exon conferred the capacity to be degraded at a rate similar to that seen in intact IRP2 upon IRP1. In order to test whether cysteines from the IRP2 specific exon were important in degradation, the degradation rate of the chimeric protein IRP1/IRP2 specific exon (IRP1+73) was compared to the degradation rate of a similar construct which differed only in that the middle three cysteines, C168, C174 and C178, of the IRP2 specific domain were simultaneously converted to serines by site-directed mutagenesis (Exon 3C-S). When the three cysteines were mutated to serines, the IRP2 specific exon could no longer transfer the capacity for rapid iron-dependent degradation to IRP1. A pulse chase experiment revealed that the exon 3C-S mutant was stable, with an estimated t1/2 of 24 h. In order to gain insight into the mechanism of degradation, iron-replete cells were treated with a variety of reagents known to interfere with various modes of proteolysis in cells. The peptide aldehyde MG132, a potent inhibitor of the 20 S subunit of the proteasome, interfered with degradation of IRP2 when cells were treated with iron. To further confirm the role of the proteasome in degradation of IRP2, a more specific inhibitor of proteasome function, lactacystin, was used. Lactacystin inhibits proteasome function by covalently binding to the amino terminal threonine of the mammalian proteasome subunit X. The effects of lactacystin in the cell are more specific for the proteasome than are those of MG132 and we found that lactacystin inhibits degradation of IRP2 at a level comparable to MG132 (Iwai et al., 1995). Use of proteasome inhibitors has permitted us to characterize intermediates in the degradation pathway. We have determined that IRP2 that is destined to be degraded is oxidatively modified as indicated by acquisition of carbonyl groups, and that the marked protein is selectively ubiquitinated.

Differences in the RNA Binding Sites of IRP1 and IRP2 may be Important in Potential Target Diversity

Using purified IRP1 and IRP2, we have performed a limited SELEX procedure and determined that there is an alternative RNA stem-loop ligand that binds to IRP1 with high affinity; the sequence of the loop is UAGUAX instead of CAGUGX. A different alternative ligand for IRP2 consists of the sequence GGGAGX. Mutagenesis of arginines important in IRE binding revealed that arginines of the active site cleft that were identified as important in the IRE binding site of IRP1 are also important in the IRE binding site of IRP2, but the pattern of dependence on each arginine differs. These data indicate that the RNA binding sites of the two IRPs differ in ways that may prove to be important. It is possible that some endogenous ligands are bound specifically by one, but not the other of the two IRPs.

Other Transcripts that are Regulated by IRP1 and IRP2

We have recently shown that the transcript of mitochondrial aconitase contains a functional IRE. However, the range of regulation is less than that seen with the ferritin IRE, and the levels of mitochondrial aconitase are decreased in animals on a low iron diet by only two-fold. The IRE of mitochondrial aconitase is less stable, and the IRE conformation may only be intermittently present, possibly accounting for the smaller degree of translational repression seen.

The Role of IRPs in Physiology

Homologous recombination has been used to "knockout" IRP1 in mice. Heterozygotes for the "knockout of IRP2 have been created. Mice lacking IRP1 only are healthy, and iron uptake and metabolism appear to be normal. However, when cells of the knockout mice are treated with hydrogen peroxide, the cells lacking IRP1 do not repress ferritin biosynthesis, whereas cells containing IRP1 do repress synthesis, most likely because of disassembly of the iron-sulfur cluster of IRP1. Breeding of the mice should enable us to assess the phenotype of mice lacking the gene products of both IRP1 and IRP2.

Cloning and Characterization of Enzymes Involved in Assembly of Iron-Sulfur Clusters in Cytosol and Mitochondria of Eukaryotic cells

Enzymes homologous to enzymes involved in assembly of iron-sulfur clusters of bacteria have been cloned in yeast and in mammalian cells. The homologues of bacterial NifS and NifU genes are localized to the mitochondria or cytosol of cells using specific 5' targeting signals. These gene products appear to coassemble with several other proteins to facilitate insertion of iron-sulfur clusters into target proteins. Yeast genetics and biochemical techniques are being used to fully characterize the molecular complex and to determine the mechanism of assembly of iron-sulfur clusters.

INTRACELLULAR METAL METABOLISM

An additional project, directed by ***Richard Klausner***, is aimed at molecular mechanisms responsible for the uptake, sensing, regulation, intracellular distribution, and utilization of toxic but essential metals using the simple eukaryote, *Saccharomyces cerevisiae*, which allows the application of powerful molecular and genetic tools to identify and characterize essential components of metal metabolism. Through this approach, a series of genes have been identified whose functions define the interlocking mechanisms by which both iron and copper are acquired from the environment by eukaryotic cells.

The objectives of this project are (1) To use the methodology of yeast genetics to identify genes involved in the regulated uptake and utilization of iron, copper and zinc, (2) to define structural and regulatory relationships among these genes, (3) to determine how cellular metals are "sensed" and how metal-dependent gene regulation ensues from this, and (4) to investigate the toxic consequences of disturbance of the iron sensing pathway. Particular attention has been given to insights that may be gained through study of the yeast homologs of human disease genes.

Identification of the Heme-Coordinating Residues Conserved between FRE1 and gp91-*phox*. Identification of a Novel Family of Membrane Cytochromes

The initial step in cellular iron acquisition by *Saccharomyces cerevisiae* (and most likely in humans) involves the reduction of iron chelates in the environment by an externally directed surface reductase. The major iron reductase of yeast is encoded by the *FRE1* gene. FRE1 bears sequence similarity to gp91-*phox*, the large subunit of the NADPH oxidase of human granulocytes, and the defective component in most patients with the childhood immune deficiency, chronic granulomatous disease. In collaboration with the laboratory of Dr. Anthony Segal, we have shown that FRE1, like gp91-*phox*, is a b-cytochrome containing hemes with low mid-point potential (about -250mv). Four critical histidine residues in the FRE1 protein of the yeast reductase (H294, H308, H364, H378) were identified by site directed mutagenesis. The members of each pair were separated from each other by 13 predominantly hydrophobic amino acids. Individual mutation of each histidine to alanine eliminated reductase activity and the entire heme spectrum. We considered that the failure to insert heme into FRE1 might destabilize the protein making it difficult to distinguish mutations affecting heme liganding from mutations affecting protein stability. However, levels of the apoprotein in the mutant strains lacking a heme spectrum were comparable to levels of the holoprotein in the wild-type strain. Therefore effects of these mutations in abrogating the heme spectrum are likely to be specific and not mediated by general effects on protein stability.

These critical residues are predicted to coordinate a bis-heme structure between transmembrane domains of the FRE1 protein. The histidine residues are conserved in the related gp91-*phox* protein of the NADPH oxidase of human granulocytes, predicting the sites of heme coordination in that protein complex which have not been previously identified. Similarly spaced histidine residues have also been implicated in heme binding by organelle cytochromes with little overall sequence similarity to the plasma membrane b cytochromes. The shared bis-heme binding motif raises the question of the evolutionary and functional relationships between the plasma membrane proteins gp91-*phox* and FRE1, and the organelle b cytochromes. At the level of the primary amino acid sequence only the spacing of the histidines in a generally hydrophobic context is conserved between these two groups of proteins. However, a more closely related protein family including FRE1 and gp91-*phox* can be defined. The members of this family possess hydropathy profiles with several (six to eight) hydrophobic domains at the amino terminus. The bis-heme binding motif occurs in this region. A hydrophilic carboxy terminal domain present in these proteins and absent in the organelle b cytochromes contains a number of conserved amino acid motifs occurring in the same order and with similar spacing, that may relate to cofactor binding (NADPH and FAD). This protein family includes the homologs of the gp91-phox gene from various other species including pigs, mice, and even plants, and these genes presumably function as part of a superoxide generating complex in cellular defense. A BLAST computer search of the recently completed *Saccharomyces cerevisiae* genome database for homologs of FRE1 identified FRE2 and seven additional open reading frames that appear to belong to the same gene family. These newly identified yeast genes resemble FRE1 to varying degrees but show little similarity to the organelle b cytochromes. The role of the bis-heme binding motif will be further clarified by definition of the cellular localization and function of the members of this gene family. It is highly likely that transmembrane electron transport will be involved in the function of these genes.

Permease-Oxidase Complex Involved in High-Affinity Iron Transport

The reduced iron generated by the activity of the externally directed reductase is assimilated into the yeast cell by the activity of a high-affinity transport system. We have reported previously on the identification of the a gene encoding the iron permease. This gene was initially called FTS3 for Ferrous Transport Suppressor, because it was identified as an extragenic suppressor of the high iron uptake phenotype of the mutant MA15 locus. However, the gene name has been changed to FTR1, for Ferrous Transporter. FET3 encodes a multi-copper oxidase which is also required for high-affinity cellular iron uptake. The phenotypes of FTR1 and FET3 mutants were noted to be similar (see previous Annual Report), and evidence for interaction between these two gene products was obtained. In order to increase iron uptake above the baseline observed in the wild-type strain, high copy-number plasmids containing both FET3 and FTR1 had to be simultaneously present. Secondly, deletion of FTR1 abrogated FET3 oxidase activity. Because the FET3 oxidase activity could be restored in vitro by addition of copper to the lysate, the defect appeared to be a consequence of failure to insert copper into the FET3 protein. This copper-reconstituted FET3 oxidase activity migrated aberrantly on a gel. However, after treatment of the extracts with endoglycosidase H, the mobility of the copper-reconstituted oxidase was indistinguishable from that of the control. An explanation for these findings could be that in the ftr1 mutant the FET3 protein is mislocalized resulting in aberrant glycosylation. In contrast, failure to activate FET3 oxidase due to copper deficiency did not result in abnormal FET3 glycosylation suggesting that FTR1 is required both for maturation of FET3 through the secretory pathway and its acquisition of copper.

Thus, effects of FTR1 expression on FET3 cellular localization were observed, and conversely, effects of FET3 expression on FTR1 localization were observed. In the presence of an intact *FET3* gene, the epitope-tagged FTR1 was visualized as a bright rim of immunofluorescence consistent with a plasma membrane localization. However, in the absence of an intact *FET3* gene, the epitope-tagged

FTR1 could be seen as an internal perinuclear ring, consistent with retention of the protein in the endoplasmic reticulum. Copper deprivation or mutation of the CCC2 gene, involved in copper delivery to FET3, did not give this pattern, but rather allowed surface localization of FTR1 protein to be achieved. In sum, then the genetics and the reciprocal effects of FET3 and FTR1 on localization suggest the existence of a protein complex at the plasma membrane. This would be consistent with the demonstrated role of the two components in high-affinity iron uptake. However, direct evidence of physical association of the components of the complex has still not been obtained.

Localization of CCC2 Gene Product by Evaluation of a Panel of sec and vps Mutants

The cellular processes affected in two human genetic disorders of copper metabolism, Menkes disease and Wilson disease, involve the export of copper into the secretory pathway by putative copper-transporting P-type ATPases, but this export activity has been difficult to characterize. The CCC2 gene product of yeast is homologous to the human ATPases and also appears to function in copper export, since CCC2 protein is required for the delivery of cytosolic copper to the FET3 gene product, a copper enzyme required for high-affinity iron uptake whose copper binding domains lie outside of the cytosol. Sec mutants with temperature sensitive blocks in the secretory pathway were labeled with radioactive copper to show that Fet3p apoprotein must exit the Golgi apparatus before it is loaded with copper. sec7, sec18, and sec23 mutants failed to insert copper into Fet3p when labeled at the non-permissive temperature, whereas sec 4 and sec1 mutants labeled normally. These results localize CCC2-dependent intracellular copper export to a post-Golgi compartment Several mutants with post-Golgi defects in vacuolar protein sorting were found to have iron uptake defects similar to those in ccc2 mutants, a finding that explains the long-recognized role of vacuolar biogenesis in mitochondrial respiratory function.

Cellular Iron Homeostasis - Role of AFT1

Iron deprivation of *Saccharomyces cerevisiae* induces transcription of genes required for high-affinity iron uptake. AFT1 mediates this transcriptional control. The 5' flanking region of *FET*3 was analyzed and found to contain a DNA sequence responsible for AFT1-regulated gene expression. AFT1 was capable of interacting specifically with this DNA sequence. *In vivo* footprinting demonstrated occupancy of the AFT1 binding site in cells deprived of iron and not in cells grown in the presence of iron. Thus, the environmental signal resulting from iron deprivation was transduced through the regulated binding of AFT1 to the *FET3* promoter, followed by activation of transcription.

A regulon of genes under the control of AFT1 could be defined. AFT1 was able to bind to a consensus binding site (PyPuCACCCPu) in the 5' region of genes involved in iron acquisition. These genes include FRE1 and FRE2, responsible for the surface iron reductase, and FET3 and FTR1, responsible for transmembrane transport of the reduced iron substrate. Interestingly, CCC2 was also found to be regulated by AFT1 and to possess an AFT1 binding site in its promoter region. CCC2 encodes a P-type ATPase required for copper delivery to FET3, which in turn is required for iron uptake. The AFT1 regulation of the CCC2 transcript is consistent with the role of this gene in iron metabolism.

Finally, a homolog of FTR1 was identified by data base searching. This gene, called FTH1 (ORF YBR207w), was found to possess an AFT1 regulatory site which was shown to interact with AFT1 protein by mobility shift assay. Moreover, the FTH1 transcript level was found to be regulated by iron and by AFT1. Iron deprivation induced FTH1 expression and iron loading repressed expression. Also, FTH1 expression was virtually undetectable in the Δaft1 strain. However, in the presence of the dominant AFT1-1up allele, iron regulation was still observed. This is in contrast with the other iron-regulated genes examined, and suggests that an additional AFT1-independent iron sensing/regulation pathway may exist. The FTH1 gene has been deleted and overexpressed, and a phenotype has not been discerned. Therefore, definition of a function for this gene must await further work.

Cellular Copper Homeostasis - Role of MAC1

The FRE1 promoter fused to a HIS3 reporter was used to select mutants which fail to repress the promoter activity. Among these mutants were dominant alleles of AFT1 and another dominant mutant, called MA20. The following phenotypes were noted for the MA20 mutant: (1) high surface reductase poorly repressed by iron (2) elevated copper uptake and (3) copper sensitivity. These phenotypes were similar to phenotypes of the previously described MAC1up1 mutant. By performing the appropriate crosses and analyzing the tetrads, the mutation in MA20 was shown to be allelic with MAC1.

MAC1 was known to be a nuclear protein, and because of the copper-related phenotypes, we considered that it might be the regulator controlling expression of CTR1, the plasma membrane copper transport protein. Consistent with this hypothesis, deletion mutants of MAC1 and deletion mutants of CTR1 exhibited similar phenotypes - lack of high-affinity iron uptake which could be corrected by addition of copper (50µM copper sulfate to YPD) to the growth medium. A candidate target sequence for the MAC1 regulator was identified by deletion analysis of the CTR1 promoter, and interestingly this sequence included a palindrome containing the nucleotides TTTGCTCA. This sequence was noted to be identical to a sequence appearing as a direct repeat in the FRE1 promoter. Finally, a gel shift assay was established using MAC1 protein synthesized in vitro. In this assay, a CTR1 oligonucleotide derived from the regulatory site was specifically shifted by the MAC1 protein. The signal could be competed by the FRE1 direct repeat sequence. Thus, MAC1 appears to be a DNA interacting protein which most likely functions to transduce a cellular copper signal to FRE1 and CTR1, genes involved in copper homeostasis. Although FRE1 appears to be regulated by both AFT1 and MAC1, CTR1 is regulated by MAC1 alone and not by AFT1, consistent with its role in copper metabolism.

Iron Intoxication: Implications for Regulation of the Cell Cycle

The human disease hemochromatosis is a genetic disorder caused by excess uptake of iron from the diet, resulting in multi-organ failure and, often, liver cancer. Although a candidate gene for this disorder was recently identified, the molecular events through which iron causes damage to cells are largely unknown. We have investigated the toxic effects of excess iron accumulation in *S. cerevisiae* by using a dominant mutant allele of an iron regulator and found evidence of iron-mediated regulation of the cell cycle. Overexpression of a dominant mutant allele of *AFT1*, *AFT1-1*^{up}, results in high levels of ferrous iron uptake that is associated with a growth arrest and decreased survival in the presence of excess iron. Deletion of a component of the high affinity iron transport complex, *FET3*, relieves the growth impairment at high iron concentrations. *AFT1-1*^{up} strains grown in excess iron arrested with a high proportion (85%) of large, unbudded cells and flow cytometry reveals an accumulation of cells with 1N DNA content, consistent with a cell cycle arrest in G_1 or early S phase. Treatment of iron-arrested cells with mating pheromone demonstrated that the small fraction of cells with 2N DNA content is resistant to alpha factor, suggesting that a cell cycle arrest in both G_1 and G_2 occurs. The G_2 block is exacerbated by deletion of the DNA repair genes *RAD1*, *RAD6*, *RAD52*, and *APN1*, and is dependent on the DNA damage checkpoint gene, *RAD9*. Expression of *AFT1-1*^{up} in strains deficient in DNA repair causes dramatic reductions in survival and markedly slowed growth. Expression of *AFT1-1*^{up} is mildly recombinogenic and mutagenic. These data provide evidence indicating that iron intoxication leads to DNA damage, which activates a cell cycle checkpoint and results in a cell cycle arrest in G_2.

The G_1 arrest associated with iron intoxication is due to down- regulation of the G_1 cyclins CLN1 and CLN2 by iron. This results in decreased activity of the CDC28 kinase and failure to progress through Start. Whether the down-regulation of G_1 cyclins occurs through alteration of protein half-life or through transcriptional regulation is an area of current research. The yeast gene GRR1 is a regulator of glucose transport and is required for efficient degradation of the G_1 cyclins. A *grr1* strain progresses rapidly through G_1 and microscopically appears with multiple elongated buds. Expression of *AFT1-1*^{up} in this strain results in a reversal of both the cell cycle and morphologic phenotypes, which is consistent with the iron-mediated down-regulation of CLN1 and CLN2. Strains expressing a hyperstable form of the G_1 cyclin CLN3 (*cln3-2*) also progress rapidly through G_1, but expression of *AFT1-1*^{up} in this strain fails to produce a cell cycle arrest. The effect of iron on CLN1 and CLN2 levels is being investigated in the *CLN3-2* strain. The G_1 arrest appears to be at least partially independent of DNA damage (it is not dependent on RAD9 and is not affected by mutations in DNA repair pathways). This raises the intriguing possibility that the eukaryotic cell cycle in regulated by iron or oxidative stress in a specific fashion. Mutant strains that demonstrate reduced survival in the presence AFT1-1up have been isolated and experiments are underway to isolate a subset of mutants that demonstrate alterations of the cell cycle phenotypes. This area of research will provide new insights as to how environmental signals are integrated into the regulation of the cell cycle.

Studies of Zinc Metabolism Using Yeast Genetics

Like iron and copper, zinc is a trace element indispensable to most organisms. Although the importance of zinc to biochemistry is well known, little is known about how cells take up and distribute this essential nutrient. Such mechanisms are likely to be homeostatically regulated, since zinc is also toxic in excess. In addition, the secondary physiological consequences of zinc deficiency and zinc toxicity are poorly understood at the biochemical level. This knowledge is especially needed for a better understanding of human nutritional zinc deficiency and its many consequences, including birth defects and immunodeficiency.

Over the past year, many of the same genetic approaches that have proved useful for the analysis of iron and copper metabolism have been extended to studies of zinc. A genetic screen for zinc-regulated genes in yeast has been carried out, based on the assumption that important genes in zinc metabolism are zinc-regulated. This assumption was in turn grounded in the knowledge that mechanisms of iron and copper uptake are transcriptionally regulated. Random lacZ fusions were introduced into the yeast genome by transposon-mediated mutagenesis, using a library of such fusions created in the laboratory of M. Snyder (Yale University). Translational fusions involving zinc-regulated genes were then identified by isolating clones exhibiting zinc-regulated lacZ expression. Several technical modifications were introduced that greatly expedited this procedure. To date, 36 zinc-regulated genes have been identified, of which 12 exhibit over 20-fold regulation by changes in extracellular zinc availability.

Current efforts are focused on gaining a clearer understanding of the function of a putative transport protein identified in the genetic screen. This protein contains six transmembrane domains and several potential metal-binding sites, and importantly, is highly induced (>100-fold) in response to zinc deprivation. Loss of function mutations appear to result in a phenotype of flocculent growth that is also characteristic of growth of wild-type strains in low-zinc medium, suggesting that this Transporter functions in zinc uptake or zinc utilization. Among the other zinc-regulated genes identified in this screen are those encoding the zinc-resistance protein Zrc1p, two zinc-finger proteins, the sodium Transporter Pmr2p, and the widely distributed yeast retrotransposon, Ty1. Tools for the systematic study of these and other genes are now being tested and are anticipated to lead rapidly to many new insights into how eukaryotic cells respond to zinc deprivation and toxicity.

Relevance to Human Physiology and Disease

The genetic analysis of the iron uptake system in yeast has continued to yield insights that may be relevant to human physiology and disease. Some of the yeast genes implicated in iron and copper metabolism are homologous to human genes. FRE1, a structural gene required for the predominant yeast iron reductase, is homologous to gp91-*phox*, a structural gene of the NADPH oxidase of human granulocytes. The residues within the human oxidase where the essential heme cofactors are bound have not been defined despite efforts by several laboratories. These residues could be identified in the yeast predicted protein, and extrapolation of this information predicts the sites of heme coordination in the human protein (see above).

The yeast CCC2 gene is homologous to the human Wilson and Menkes disease genes. Localization of the yeast gene product (see above) may have implications for the mechanism of action and the cellular site of action of the Menkes and Wilson disease gene products.

The CCC2 protein functions in the delivery of the copper to FET3, a multi-copper oxidase required for iron uptake. Analogously, the Wilson disease gene functions in the delivery of copper to ceruloplasmin. Ceruloplasmin has been implicated in iron metabolism in humans, and inactivating mutations in this gene lead to iron accumulation in the basal ganglia. Many questions remain, however. An iron import complex such as the FET3/FTR1 complex proposed for *Saccharomyces cerevisiae* has not been described in humans. Moreover the problems resulting form copper deficiency or inactive ceruloplasmin relate primarily to export of iron from the cells. It is possible that ceruloplasmin could interact with distinct membrane components to mediate export and/or import of iron. Alternatively, a novel multi-copper oxidase might function in iron uptake as part of a complex with a still unidentified permease.

We have begun to look for the human homolog of the FTR1 gene. Homologs in *B. subitilis* and *S. pombe* have been identified by DNA sequencing, although there is no biochemical data on the corresponding genes. A homologous gene in *S. cerevisiae* (*FTH1*) was identified as part of the genome project, and we have studied the iron-regulated expression of this gene (see above), although the deletion strain has no apparent phenotype. As part of a collaboration with the laboratory of Dr. Gerald Fink, we have used a library of genomic DNA from *Candida albicans* to complement the *FTR1* deletion strain of *S. cerevisiae*. The sequence of the complementing clone contains an ORF with more than 50% amino acid identity with the FTR1 protein. Comparison of the sequences of these FTR1 homologs and data from site directed mutagenesis of a motif (REGLE) suggested that this conserved sequence is critical for the iron transport function of FTR1.

Approaches to identifying the human homolog of FTR1 include 1) PCR based on degenerate sequences from conserved regions of FTR1 predicted protein and 2) functional complementation of the growth defect of the FTR1 mutant on iron depleted (ferrozine) plates. For this purpose, a cDNA library is being generated for insertion into a yeast expression vector under the control of the powerful PGK promoter.

The putative zinc transport protein identified in yeast contains structural motifs that appear to be conserved in several human homologs, including distinct variants isolated from liver and placenta. As nothing is presently known about the molecular basis of zinc transport in humans, these homologs will obviously receive careful scrutiny in the near future. A major potential application of this work is the development of sensitive diagnostic markers for zinc deficiency, analogous to the use of transferrin receptor assays for determination of iron status. Such definitive markers are currently lacking in the field and can be expected to have substantial clinical utility, given the wide prevalence of zinc deficiency in diarrheal diseases, malnutrition, and pregnancy. Studies of the pathophysiology of zinc deficiency and zinc toxicity in yeast may also provide heuristic guidance for analogous studies in human tissues.

GENE REGULATION IN RESPONSE TO ENVIRONMENTAL STRESS

A third group, directed by ***Gisela Storz***, studies how organisms sense environmental signals and transduce the signals into changes in gene expression cell morphology. The group has continued to characterize the bacterial and yeast response to hydrogen peroxide. *Arabidopsis* is used to study how plant development is regulated by light.

Oxidative Stress Responses in Bacteria and Yeast

Reactive oxygen species ($O_2\bullet^-$, H_2O_2, and HO•) can lead to the damage of almost all cell components (DNA, lipid membranes, and proteins) and have been implicated as causative agents in several degenerative diseases. Most organisms have an adaptive response to defend against oxidants. Treatment of both bacterial and yeast cells with low doses of H_2O_2 results in the induction of distinct groups of proteins, the decreased expression of other proteins, and increased resistance to killing by subsequent higher doses of H_2O_2. In bacterial cells, a key regulator of the defenses against H_2O_2 is the OxyR protein. OxyR appears to be both the sensor and transducer of the oxidative stress signal, since the oxidized but not the reduced form of the purified regulator can activate transcription *in vitro*. H_2O_2 treatment also induces the expression of OxyS, a unique, non-translated RNA which has been shown to activate and repress as many as 40 genes in *E. coli*. The mechanism of OxyS action is now under investigation. Less is known about the induction of eukaryotic defenses against H_2O_2, but we have recently initiated studies to identify regulators of the *S. cerevisiae* response to oxidative stress.

Characterization of the Redox-Active Center of OxyR

We previously isolated several non-binding and constitutively-active OxyR mutants. We are now studying the biochemical properties of wild type OxyR together with the mutant proteins to elucidate the nature of the redox-active center in OxyR. Metal analysis and experiments in which OxyR was denatured, dialyzed, and renatured, suggest that OxyR does not contain a metal co-factor. Therefore, we favor the hypothesis that the redox-active center of OxyS is Cys199 which is oxidized to a sulfonic acid upon exposure to H_2O_2. The status of this cysteine residue is now being probed with mass spectrometry and chemicals shown to react specifically with sulfonic acid. In addition, we have initiated studies to identify factors that re-reduce the OxyR protein in the cell.

Regulation by the OxyS RNA

We are continuing our studies of the novel, 109 nucleotide RNA which is induced by H_2O_2. Previously, we found that OxyS acts in *trans* to activate and repress the expression of a number of genes including *fhlA*, which encodes a transcriptional activator, and *rpoS*, which encodes a σ subunit of RNA polymerase. To gain insights into the mechanisms of OxyS action, we examined OxyS repression of *fhlA* and *rpoS*. OxyS acts at a post-transcriptional level for both of these genes and probably prevents *fhlA* translation by pairing with mRNA sequences adjacent to the ribosome binding site. The OxyS response site at *rpoS* has not been identified, but we have recently found that OxyS-mediated repression requires HF-I, a non-specific RNA binding protein. We are now testing whether OxyS binds HF-I or any other proteins. In parallel studies, we compared the levels of OxyS with the levels of other small stable RNA species and found that OxyS is abundant with as many as 4,000 molecules of OxyS per cell.

rpoS expression is regulated at multiple levels, and in the course of our studies of OxyS-mediated repression, we isolated a mutation in *rssB* which stabilized the RpoS protein. Interesting, RssB shows homology to response regulators and is the first such regulator shown to be involved in the control of protein turnover.

Regulators of the Yeast Response to Oxidative Stress

Relatively little is known about the eukaryotic defenses against oxidative damage. Therefore, we have also begun to examine the *S. cerevisiae* response to H_2O_2. To gain an overview of what known regulators are important for protection against H_2O_2, we constructed isogenic strains carrying mutations in *YAP1*, *YAP2*, *MAC1*, *POS9*, *MSN2*, and *MSN4*. We are comparing the viability of these mutant strains under different stress conditions and are examining the induction of antioxidant genes in the mutant strains. We have also begun to use the mutant backgrounds to isolate suppressor mutations in additional regulators and additional components of the oxidative stress signal transduction pathways.

Blue Light Regulation in *Arabidopsis*

The normal development of plants is dependent on light, and spectral studies have shown that plants undergo morphological changes in response red/far-red, blue, and UV light. For example, dark grown plants have extremely elongated hypocotyls (stems) compared to seedlings grown under red or blue light. At present, the mechanisms by which the light signals are transduced into changes in plant growth and morphology are largely unknown.

Characterization of *hy4* and *hy5* Mutants

We took advantage of the light-effect on hypocotyl length to screen for *Arabidopsis* mutants that have elongated hypocotyls under blue light. 24 independent mutants defective in their developmental response to blue light were isolated from a screen of 300,000 fast neutron-mutagenized seeds. The mutants fell into two complementation groups corresponding to the *HY4* and *HY5* genes. Since *HY4* is thought to encode the blue light photoreceptor, we characterized the 21 *hy4* mutants to determine the nature of the mutations. We found 20 of these fast neutron-generated mutants displayed straightforward genetic behavior and had detectable mutations at the *HY4* locus. Many of the mutations were large deletions demonstrating that fast neutron irradiation is an effective means of generating deletions suitable for genomic subtraction. The last *hy4* mutant showed extremely interesting genetic behavior; the strain exhibited a wild type phenotype in one generation and a mutant phenotype in the next. Since we have not been able to detect a molecular defect at the *HY4* locus, we propose that this line has an epigenetic mutation, the first such mutation to be identified in *Arabidopsis*. These studies of the *hy4* mutants are being complemented by subtractive screens between wild type, *hy4*, and *hy5* cDNA libraries to identify genes that are differentially expressed between the wild type and mutant seedlings.

REGULATION OF THE FATE OF NEWLY SYNTHESIZED PROTEINS IN THE CENTRAL VACUOLAR SYSTEM

A group headed by ***Juan Bonifacino*** studies the regulation and the fate of newly synthesized proteins that enter the central vacuolar system. The interior of the cell is organized into a diverse array of membrane-bound compartments. Each of these compartments is characterized by the presence of a specific set of resident proteins. It is now well established that the localization of integral membrane proteins to the various compartments of the endocytic and secretory pathways is mediated by information encoded within the structure of the proteins. In some cases, targeting information is contained within short, linear sequences of amino acid residues that act as specific sorting signals; the signals are recognized by receptor-like molecules. In other cases, the targeting information is a manifestation of a global physical-chemical property of the proteins, such as a tendency to partition into membranes with a particular lipid composition. Over the past year, our group has conducted studies on both types of mechanisms. Most of our work has focused on tyrosine-based sorting signals involved in internalization from the cell surface and lysosomal targeting and on adaptor complexes that function as recognition molecules for such signals. We have also studied the role of transmembrane domains in protein retention within the endoplasmic reticulum (ER), which may be mediated by lipid partitioning. Our recent progress on these projects is described in the following sections.

Molecular Mechanisms Involved in Sorting Integral Membrane Proteins to Endosomes and Lysosomes

Many integral membrane proteins contain within their cytoplasmic tails tyrosine-based sorting signals that conform to the tetrapeptide motif YXXØ, where Y is tyrosine, X is any amino acid, and Ø is an amino acid with a bulky hydrophobic side chain. These signals mediate various sorting processes, including internalization from the cell surface and lysosomal targeting. We had previously demonstrated that the medium chains, μ_1 and μ_2, of the clathrin-associated adaptor complexes, AP-1 and AP-2, function as the recognition molecules for tyrosine-based sorting signals. AP-2 is found in association with the plasma membrane and mediates internalization from the cell surface, whereas AP-1 localizes to the TGN where it mediates targeting of newly synthesized proteins to lysosomes. The involvement of tyrosine-based sorting signals in more than one sorting process raises the question of how tyrosine-based signals that conform to the same consensus motif can mediate sorting to different end compartments. In order to address this question we have examined the factors that determine the fine specificity and the avidity of signal-adaptor interactions. These studies have demonstrated that: (a) in general, μ_2 binds tyrosine-based signals with higher avidity than μ_1, (b) μ_1 and μ_2 display different preferences for residues surrounding the critical tyrosine residue, and the © position of the signal within the cytoplasmic tail of the proteins is critical for interactions; for instance, placement of the signal at the carboxy-terminus of the tail greatly increases the affinity of both μ_1 and μ_2 for the tyrosine-based signals. Thus, it is the combination of the sequence of the signal and its position within the tail that determines the overall strength of interaction with μ_1 and μ_2. Based on these findings, we have proposed a model according to which strong signals will bind to μ_1 in the TGN, resulting in lysosomal targeting. Weak signals, on the other hand, will not be recognized by μ_1, thus allowing for transport of the proteins to the cell surface. Once at the surface, the proteins will then be in a position to bind to the high affinity μ_2, leading to their internalization into endosomes.

Sequence Requirements for Interaction of Tyrosine-Based Sorting Signals with the Adaptor Medium Chains Determined by Combinatorial Methods

The identification of the medium chains, μ_1 and μ_2, as the receptors for tyrosine-based sorting signals has enabled analyses of the properties of the signals at a molecular level. In order to establish the sequence requirements for interaction, we screened combinatorial libraries using μ_2 as a selector molecule. One of the approaches consisted of screening a combinatorial DNA library using the yeast two-hybrid system. In another approach, carried out in collaboration with the groups of Tomas Kirchhausen and Lewis Cantley (Harvard Medical School), combinatorial peptide libraries were screened *in vitro* with recombinant μ_2. Both approaches resulted in the identification of the sequence YXRL as the optimal signal for interaction with μ_2. Analyses of suboptimal sequences revealed that all of the selected peptides conform to the YXXØ motif defined previously from studies in vivo. We also examined the effect of mutations at the four positions on interactions with μ_2 by surface plasmon resonance spectroscopy. We found an excellent correlation between the apparent affinity of different signals for μ_2 *in vitro* and their sorting activity *in vivo*. These observations confirm the role of μ_2 as a receptor for tyrosine-based sorting signals and reveal additional properties of the interaction.

In vivo Overexpression/Saturation Experiments Suggest that Tyrosine- and Di-Leucine-Based Sorting Signals Utilize Different Components of the Sorting Machinery

During the past year, we completed our studies on the saturability of signal-mediated protein sorting *in vivo*. The existence of specific recognition molecules that bind to tyrosine-based sorting signals predicts that processes mediated by such signals should be saturable. We tested this prediction by overexpressing proteins that have tyrosine-based signals and analyzing the surface expression of endogenous proteins that are normally targeted to lysosomes by virtue of tyrosine-based signals. We observed that overexpression interfered with several processes dependent on tyrosine-based signals such as lysosomal targeting and internalization from the cell surface. Interestingly, overexpression of proteins containing di-leucine-based signals, which are also involved in internalization and lysosomal targeting, did not compromise sorting mediated by tyrosine-based signals but did interfere with the sorting of other proteins containing di-leucine-based signals. These results suggest that tyrosine-based and di-leucine-based signals utilize distinct saturable components of the sorting machinery. Since tyrosine-based signals are known interact with μ_1 and μ_2, our findings most likely imply that di-leucine-based signals interact with either another subunit of the adaptor complexes or with a different type of protein. These studies were extended to the HIV-1 envelope glycoprotein complex, which also has several tyrosine-based signals that are active in endocytosis. Overexpression of the envelope glycoprotein, and of chimeric proteins derived from it, resulted in missorting of lysosomal proteins to the cell surface, a phenomenon that may explain some of the cytopathic effects of HIV-1 on the host cells.

A Novel Adaptor-Like Complex that Recognizes Tyrosine-Based Sorting Signals

In addition to internalization and lysosomal targeting, tyrosine-based signals have been implicated in various other sorting processes, including transport from early to late endosomes, delivery to specialized endosomal/lysosomal organelles such as MHC class II antigen-processing compartments, localization to the TGN, and targeting to the basolateral surface of polarized epithelial cells. The diversity of the processes mediated by tyrosine-based sorting signals makes it difficult to explain the function of the signals solely on the basis of interactions with the AP-2 and AP-1 adaptors. It is thus conceivable that there may be additional adaptor-like complexes that mediate some of the additional sorting functions attributed to tyrosine-based signals.

We have recently identified a novel adaptor-like complex that is a likely candidate for being another receptor for tyrosine-based sorting signals. The identification of this complex was made possible by the cloning of two novel cDNAs encoding proteins that have ~30% sequence identity to the adaptor small chains σ_1 and σ_2. The two proteins, which were named σ_{3A} and σ_{3B}, are very closely related to each other and are expressed in all cells and tissues examined. An antibody that recognizes both σ_3 proteins immunoprecipitates from fibroblasts a complex with a subunit composition similar to AP-1 and AP-2. The medium chain of this novel complex, known as p47A, also interacts with tyrosine-based signals. Experiments are now underway to establish the localization of this complex within cells; preliminary evidence suggests that it may associated, at least in part, with endosomes. We speculate that this novel complex might be involved in sorting proteins from early to late endosomes or from endosomes to the basolateral plasma membrane of polarized cells.

Role of Transmembrane Domains in Protein Sorting

All of the studies described above dealt with sorting events mediated by interactions between signals and their recognition molecules. Some sorting processes, however, do not rely on signals but depend on some physical-chemical property of the proteins. Several years ago, we demonstrated that transmembrane domains that either had charged or strongly hydrophilic amino acids or were too short caused retention of incompletely assembled proteins in the ER. In collaboration with Allan Weissman (NCI), we have now extended these observations to the ER resident protein UBC6. UBC6 is a cytosolically-oriented ubiquitin-conjugating enzyme attached to the ER membrane by a carboxy-terminal hydrophobic anchor. Molecular dissection of UBC6 revealed that the membrane anchor was both necessary and sufficient for ER localization. Moreover, we demonstrated that lengthening the hydrophobic span of the membrane anchor caused release from ER retention and allowed transport to the plasma membrane via the classical secretory pathway. Thus, we conclude that the carboxy-terminal membrane anchor of UBC6 is responsible for the retention of the protein within the ER and that the length of the transmembrane span is what determines its role in ER retention. We speculate that the mechanism by which the UBC6 membrane anchor mediates ER retention is related to that involved in protein localization to the Golgi complex. A current view of this mechanism is that short transmembrane domains may cause partitioning of proteins into the thinner membranes of the ER and the Golgi complex rather than into the thicker membranes of post-Golgi organelles.

LOCALIZATION AND DYNAMICS OF INTRACELLULAR ORGANELLES

A second group studying cell biology, led by ***Jennifer Lippincott-Schwartz***, has focused on the control of the organization and distribution of organelles and transport intermediates within the endomembrane system of higher eukaryotes. Over the past year this group has focused on understanding the dynamics of membrane proteins which comprise the ER/Golgi membrane system, which plays a central role in the biosynthesis, transport, processing and sorting of secretory products within the cell. Toward this goal, protein chimeras containing the green fluorescent protein (GFP) have been generated which localize within the ER/Golgi membrane system and have been used to examine the dynamics and diffusion rates of these proteins in interphase and mitotic cells.

Probing the Diffusional Mobility of Golgi Protein-GFP Chimeras

The Golgi complex contains a large number of resident components that play important roles in membrane processing and sorting. How these proteins are retained in the Golgi despite a continuous flow of protein and lipid through the secretory pathway is not yet understood. To address this question the diffusional mobilities of mammalian Golgi membrane proteins (including galactosyltransferase, mannosidase II and KDEL receptor) fused to GFP were measured in living HeLa cells. This work utilized fluorescence photobleaching recovery (FPR) and fluorescence loss in photobleaching (FLIP) techniques to measure the diffusion rates of GFP-chimeras in Golgi membranes and to examine the extent of lateral membrane continuity between Golgi stacks. The results from this work revealed rapid and extensive diffusion of all GFP-Golgi chimeras within Golgi membranes, indicating Golgi retention of these proteins does not depend on protein immobilization. Rapid and widespread loss of fluorescence from the Golgi upon repeated bleaching of a small zone within the complex, furthermore, implied significant lateral diffusion rather than vesicular transport of the GFP-tagged proteins between Golgi stacks. These results raise the question of how Golgi membranes maintain their identity amidst rapid diffusion of resident components and what role such dynamic properties play in Golgi membrane structure and function.

Diffusional Mobility of Golgi Proteins in Mitotic Cells

The Golgi apparatus undergoes a profound reorganization during mitosis in many cell types and is widely assumed to vesiculate into thousands of small vesicles scattered throughout the cytoplasm, which remain inactive until the end of mitosis when they actively fuse to regenerate Golgi stacks. Ultrastructural studies of mitotic cells have supported this thinking by showing Golgi proteins in vesicle clusters. However, Golgi proteins have also been observed in the continuous membranes of the ER during mitosis, raising the possibility that these proteins are not restricted in their localization to static vesicles. To investigate this possibility the diffusional mobility of Golgi proteins tagged with GFP were examined in mitotic HeLa cells. Using confocal microscopy a narrow box across the edge of a mitotic cell expressing galactosyltransferase (Galtase)-GFP was repeatedly bleached with high power laser light and areas outside the box were monitored for fluorescence loss in photobleaching (FLIP). Galtase-GFP fluorescence associated with the entire cell was lost with a half-time of 2 min indicating that all of the chimeras were highly mobile and could diffuse rapidly into the narrow photobleached zone. The rate of loss of Galtase-GFP fluorescence was significantly faster than that of rhodamine transferrin or rhodamine dextran observed by FLIP, and resembled that of ER-localized GFP chimeras. These results suggest that Golgi proteins in mitotic cells are not restricted in their localization to static vesicles, but are diffusing rapidly within an interconnected membrane system, which most likely includes the ER.

Kinetics of Brefeldin A-Induced Resorption of Golgi into ER: Evidence of Flow-Assisted Membrane Transport

GFP-Golgi chimeric proteins were used to examine the dynamics of the Golgi complex in vivo and how it is perturbed by brefeldin A (BFA). BFA dramatically alters the distribution and transport of membrane through organelles by blocking an essential activation step in the membrane interaction of a family of closely related proteins called ADP ribosylation factors, which are low molecular weight GTP-binding proteins. In control cells imaged at 3 sec time intervals with a confocal microscope system, thin membrane tubules were found to rapidly extend out and disconnect from stable perinuclear Golgi elements. Detached, mobile tubule elements containing the GFP chimeras moved away from the Golgi along curvilinear microtubule tracks at rates of 0.6 um/sec before changing shape and direction. Upon BFA-treatment, tubule processes appeared more frequently and failed to detach from Golgi structures. A dynamic tubule network extending through the cytoplasm was soon generated and persisted for 5-10 min until rapidly emptying its contents into the ER within 15-30 sec. Both lipid and protein emptied from the Golgi at similar rapid rates leaving no Golgi structure behind. The kinetics of the redistribution process was followed and compared with numerical simulations of diffusion in two dimensions that take account of the actual Golgi shape and the observed density of ER membrane. A large discrepancy between simulation and experimental data was found suggesting an alternative model for Golgi redistribution into the ER that is analogous to wetting or absorption phenomenon where a membrane flow supplements diffusive transfer. Such flow-assisted redistribution of Golgi into ER during BFA treatment suggests that mechanisms which regulate retrograde tubule formation and detachment from the Golgi are integral to the existence and maintenance of this organelle.

ER-to-Golgi Transport of VSVG-GFP Chimeras in Living Cells

The nature of the transport intermediates that carry proteins between the ER and Golgi complex is currently unclear. Two major models have been proposed. In one, proteins move through a long-lived 'intermediate compartment' which continually packages new transport vesicles for transport into the Golgi complex. In the second, cargo-containing vesicles bud from the ER and fuse to form larger pleiomorphic structures (i.e. intermediate compartment) which themselves translocate to and fuse with the Golgi. To distinguish between these models, the ts045 mutant of VSVG protein was tagged with GFP (VSVG-GFP) and expressed in Cos cells. VSVG-GFP has the property of misfolding and being retained in the ER at 40°C. This property is rapidly reversible upon shift to 32°C, permitting the release of a synchronized pool of protein to the Golgi complex. ER-to-Golgi transport of VSVG-GFP was followed using time-lapse imaging of cells on a fluorescent microscope equipped with a cooled CCD camera and filters optimized for GFP. VSVG-GFP accumulated in tubulovesicular structures (TVS) scattered throughout the cell with shift from 40°C to 16°C for two hours. These TVS resembled structures previously described as the intermediate compartment and contained ßCOP. Upon shift from 16°C to 32°C, TVS translocated rapidly as units into the centrosomal region and fused with the Golgi complex. TVS moved along straight or curvilinear paths at speeds up to one micron per second, consistent with a microtubule minus end motor powering their movement. A single long tubule was often seen extending in the direction of motion from a motile TVS, suggesting that a motor on the tip of the tubules was pulling the TVS towards the Golgi. This motility did not occur after microtubule disruption with nocodazole. Upon shift directly from 40°C to 32°C similar fast movement of TVS from peripheral sites into the centrosomal region was observed. These results favor a model for ER-to-Golgi transport in which the intermediate compartment itself translocates to and fuses with the Golgi complex.

Golgi-to-ER Transport of VSVG Chimeras

Proper functioning of the secretory pathway in higher eukaryotes requires a significant retrograde flow of membrane and protein from the Golgi complex back to the endoplasmic reticulum (ER). This ensures return of escaped ER resident proteins, as well as membrane components necessary for continued anterograde traffic. A variety of molecules have been shown to cycle constitutively between the ER and Golgi complex, although the exact routes these molecules travel is unclear. Whether Golgi resident proteins cycle through these pathways, as well, is unknown. To address this question we constructed chimeric Golgi proteins containing the luminal domain of the temperature sensitive viral glycoprotein, tsO45VSV G. We reasoned that if Golgi-targeted VSV G chimeras were to cycle between the ER and Golgi complex, then shifting the temperature to 40°C should result in their misfolding and retention in the ER as they cycled through this compartment. In transiently transfected cells at 32°C, VSVG chimeras (including VSV G attached to the transmembrane and cytoplasmic domains of TGN 38, KDELR and KDELR_m) were found localized to the Golgi complex. Upon shifting the temperature to 40°C, these proteins redistributed into the ER, without altering the distribution of other Golgi membrane markers. The kinetics of this process varied with each chimera, with complete redistribution occurring in as little as fifteen minutes, or as long as three hours. This process was fully reversible, as all chimeras showed a Golgi staining pattern within one hour of shift back to the permissive temperature. These observations suggest that there is extensive retrograde traffic of Golgi membrane proteins from the Golgi back the ER.

SIGNAL TRANSDUCTION

This group, headed by ***Lawrence Samelson***, explores the biochemical basis of T cell activation in both the human and murine systems. This group has been particularly interested in the engagement of the multi-component antigen receptor in T cells (TCR), which results in the rapid activation of one or more protein tyrosine kinase pathways.

The T Cell Receptor Tyrosine Kinase(s):

Identifying the kinase(s) responsible for tyrosine phosphorylation of the TCR and the other cellular substrates has been a major goal of this Section for many years. A critical tyrosine kinase activated by the T cell receptor is the ZAP-70 protein, which is tyrosine phosphorylated and associates with the TCR upon activation. In the past year we developed reagents and methods enabling us to obtain large quantities of pure ZAP-70 for analysis. Recombinant human ZAP-70 was overexpressed in insects cells after infection with a ZAP-70-containing baculovirus. Detergent-soluble extracts were subjected to dye affinity resins and sizing columns. Analysis by SDS-PAGE revealed one 70kD band, which was reactive with ZAP-70 specific reagents. The enzyme in this preparation is active. It was used to demonstrate a requirement for Mn^{++} and a pH optimum of 6.5. Using the cytoplasmic fragment of band 3 (cfb3) as an in vitro substrate, we demonstrated an apparent K_m for ATP of 3.0μM. Kinetic values were also obtained for substrate phosphorylation. We also found that ZAP-70, unlike Lck, had a very restricted capacity to phosphorylate proteins in vitro. Only cfb3 and tubulin were phosphorylated by active ZAP-70, whereas many more examples of substrates for Lck were demonstrated. We also tested the ability of ZAP-70 to phosphorylate the tyrosine residues found in the tails of TCR subunits. These tyrosines, after phosphorylation by Lck or Fyn serve as binding sites for the SH2 domains of ZAP-70. In vitro we confirmed that recombinant Lck could phosphorylate peptides with these sites. However, ZAP-70 was unable to catalyze phosphorylations of these tyrosines. In a final experiment for this study we asked whether phosphopeptide binding to ZAP-70 would lead to enzymatic activation. It has been suggested that such binding would serve as an allosteric activation event for this enzyme. We were unable to demonstrate this effect. As described below, all evidence suggests that ZAP-70 is regulated by phosphorylation.

With collaborators in Vancouver and Seattle we previously mapped the sites of tyrosine phosphorylation of the ZAP-70 kinase. Residues Y292, Y492 and Y493 become phosphorylated after TCR engagement. These sites were then analyzed by site directed mutagenesis and expression in COS cells. In this earlier study we demonstrated that Y493 was absolutely required for kinase activation, most likely because it serves as the site of Lck phosphorylation. The mutation of Y492 to phenylalanine gave the unexpected result of leading to a four-fold increase in kinase activity. We saw no effect of a Y to F mutation at Y292 after testing in COS cells. More recently we have obtained a mutant form of the Jurkat T cell line that lacks ZAP-70. TCR engagement of this cell fails to lead to cellular activation, as there is minimal induction of tyrosine phosphorylation, no calcium flux and no lymphokine production observed. Reconstitution of the cells with ZAP-70 results in restoration of TCR-coupled signaling. Reconstitution with the various Y to F mutants was then performed. As expected the Y493F mutation failed to signal in the T cells. The Y492F and the Y292F versions of ZAP-70 both led to prolonged activation of tyrosine phosphorylation and enhanced lymphokine production as measured by IL-2 promoter transcriptional activation. These data confirm that ZAP-70 function is regulated by its tyrosine phosphorylation. One likely explanation of the significance of the phosphorylation at Y292 and Y492 is that they serve as binding sites for proteins that regulate function.

The Tyrosine Phosphorylation Pathway in T Cells:

In previous studies we have demonstrated that activation of the TCR results in phosphorylation of the TCRζ chain and multiple intracellular substrates. The identity of the other substrates that are tyrosine phosphorylated upon TCR activation has been a major question for this Section for some time. One substrate of TCR-mediated tyrosine kinase activation is the protooncogene $p120^{cbl}$, which is rapidly tyrosine phosphorylated upon T cell activation. We showed earlier that the SH3 domains of the linker molecule Grb2 binds to $p120^{cbl}$ in vitro. More recently we have demonstrated that a Grb2-$p120^{cbl}$ complex can be found in the cell. The complex dissociates upon TCR engagement with kinetics suggesting that the tyrosine phosphorylation of $p120^{cbl}$ induces the dissociation. However more extensive analysis suggests that additional factors are responsible, since dephosphorylation does not induce reassociation. The complex of Grb2 and $p120^{cbl}$ was further studied by mapping the interaction of the proteins. $p120^{cbl}$ contains a 200 amino acid region with multiple potential SH3 binding sites. Our analysis demonstrates that a construct (1-528) containing the sequence PPVPPR binds Grb2. However increased binding can be detected in longer constructs containing additional proline-rich sequences. Multiple proline rich sites could serve as alternate sites of interaction or aid in generating a structure better able to interact with SH3 domains.

The function of p120^{cbl} was also studied in RBL-2H3 cells which can be activated by engagement of the FcεRI. We demonstrated that in this system p120^{cbl} is also tyrosine phosphorylated upon receptor engagement. Using recombinant vaccinia viruses to overexpress various protein tyrosine kinases, we demonstrated that Syk, a kinase closely related to ZAP-70 is responsible for p120^{cbl} tyrosine phosphorylation in RBL-2H3 cells. In addition we mapped the site of p120^{cbl} tyrosine phosphorylation to the distal end of the molecule. We found that p120^{cbl} and Syk form a complex in RBL-2H3 cells. This too was analyzed by overexpression. The tandem SH2 domain of Syk was sufficient to bind residues 1-655 of p120^{cbl}. Interestingly we also found that the p120^{cbl} found within this complex was not tyrosine phosphorylated. This result suggests that there may be functional significance to the interaction of p120^{cbl} with the Syk kinase.

TUMOR SUPPRESSOR GENE

A project headed by ***Richard Klausner*** is directed at understanding a novel tumor suppressor gene, VHL, and the biochemistry and cell biology of the VHL gene product.

Biochemistry of the VHL Protein

We have continued our characterization of the interaction between the VHL protein and other cellular components. Studies with stable cell lines expressing epitope-tagged VHL strongly support the notion that VHL exists as a stable heterotrimer bound tightly to one molecule each of Elongin B and Elongin C. The majority of naturally occurring exon 3 mutations map to the Elongin interaction site previously defined and no other mutations or truncations seem to affect binding with the BC subunits. In order to identify other potential interacting proteins, we utilized recombinant VBC epitope-tagged complexes in order to affinity purify additional cellular interactors. Such an approach produced a series of proteins that were specifically bound to the VBC complex. Extensive biochemical studies convinced us that a set of proteins between 70 and 80 kD were specifically bound to VBC and were part of an endogenous cellular complex. These proteins were purified and peptide sequences were obtained. One of the proteins has recently been identified as a homolog of a newly recognized family proteins called cullins. CUL-1 was first reported in June 1996, as a C elegans gene that is required to maintain differentiated cells in the G_0 state. Sequence homology suggested at least six human homologs to CUL-1 in the database. These are referred to as CUL-1 through 6. The 76 kD VBC interacting protein that we identified is human cul-2. The ability to bind cul-2 is restricted to VBC complexes and preliminary data suggests that alterations in exon 1 or 2 of VHL may interrupt this interaction, in contrast to the interaction with B and C. The cullins are homologous to the *S. cerevisiae* CDC53 gene product which is also involved in cell cycle regulation and a recent report strongly suggests that CDC53 encodes an E3 ubiquitin conjugating enzyme.

An additional approach to the identification of VBC interacting proteins is to utilize phosphorylated VBC after modification of the VHL protein with a protein kinase phosphorylation site sequence and to use the phosphorylated VBC complex as a probe in a far Western blot of proteins derived from cell lines. Such an approach has identified two other strongly interacting proteins which we are now attempting to clone by an expression approach.

VHL and the Regulation of Gene Expression

In order to understand how the VHL gene product works in cells, we sought to identify potential target genes that might be regulated by VHL expression. We had demonstrated that when human kidney cancer cell lines were assayed for tumor genicity in nude mice, tumorogenic potential could be inhibited by stable retroviral transduction of the cell lines with wild-type, but not mutant, VHL. In looking at the histology of the resulting tumors, we were struck by the lack of vasculature and cellular infiltrate in the small tumor that arose from the VHL wild-type expressing cells. These cells grow normally in culture and the disparity between *in vitro* cell growth and tumor formation suggested that VHL was regulating genes that relate to the ability of the tumor cells to spread, invade or metastasize. When the tumors were stained for an angiogenic factor called vascular endothelial growth factor (VEGF), we noticed that the parental tumor line or the tumor line expressing mutant VHL made easily detectable VEGF protein. In contrast, the cell line transduced with wild-type VHL made no detectable VEGF protein. Supernatants of these different cell lines were used to test for the ability to stimulate proliferation of bovine endothelial capillary cells. Mitogenic activity was found for all the cells lines but was consistently significantly higher in the parental and mutant VHL tumor lines than in the wild-type transduced line. All of the additional mitogenic activity for these vascular endothelial cells could be inhibited by neutralizing antibodies against VEGF. We next demonstrated that the wild-type cell lines had very low levels of VEGF mRNA compared to the parental line or a line transduced with a mutant VHL. We looked to see whether this difference in the level of VEGF mRNA could be ascribed to altered rates of transcription or transcription elongation. For this purpose nuclear run-ons were performed using probes from different regions of the transcript. Despite significant differences in VEGF mRNA levels, there was no difference in the rate of transcription or in the profile of transcription activity across the transcript, strongly suggesting that VEGF mRNA control is not due to either transcription or transcription elongation but rather is most likely due to the regulation of mRNA half-life. We additionally noted that the diminution of VEGF mRNA levels in the cells expressing wild-type VHL was conditional. When the cells were stressed by being grown in low serum conditions, VEGF mRNA levels rose to that equal to those seen in the parental cell line. In other words, the presence of VHL in the cell allows the cell to regulate VEGF mRNA levels, most likely at the level of mRNA stability, in response to extracellular signals.

Cell Biology of the VHL Gene Product

Our initial studies on the localization of epitope-tagged VHL in a variety of cells demonstrated a puzzling pattern in which some cells had predominantly nuclear and others cells predominantly cytoplasmic VHL localization. We have extensively studied this phenomenon. Examination of a variety of cell growth conditions suggested that confluency profoundly affected the distribution of VHL as determined by indirect immunofluorescence microscopy. When cells were grown to confluent conditions, the distribution of VHL was largely cytoplasmic with varying degrees of nuclear exclusion. In contrast, in cells grown to very sparse conditions, the VHL protein was largely confined to the nucleus. Even with indirect immunofluorescence, this was not an all or none effect. Mutational analysis of the VHL protein demonstrated that sequences in the first exon were required for nuclear localization. This region contains a classic bipartite basic nuclear localization signal. Exon 3 mutations, particularly those that result in loss of interaction with Elongin B and C resulted in predominantly nuclear staining, regardless of the cell growth conditions. These findings became easier to interpret by looking at the distribution of VHL protein in living cells. This was accomplished by making fusions between the VHL protein and the green fluorescent protein (GFP). We demonstrated that the VHL-GFP fusion protein bound to Elongin B and C in a manner identical to that seen for the wild-type VHL. Furthermore, VHL-GFP reproduced the ability of VHL to regulate VEGF mRNA expression strongly suggesting that the fusion protein was functional and normal. Examination of the distribution of VHL-GFP in cells in culture under different growth conditions gave us somewhat different results than the indirect immunofluorescence. While confluent cells had more cytoplasmic than nuclear staining, sparse cells showed a significant amount of cytoplasmic staining, much more than had been seen by indirect immunofluorescence. The indirect immunofluorescence results could be reproduced by treating the cells expressing VHL-GFP under the same conditions used to prepare cells for immunofluorescence. This included cross-linking with formaldehyde and permeabilization with saponin. The cross-linking resulted in no effect on the distribution of VHL-GFP. However, when the cross-linked cells were permeabilized, the cytoplasmic staining seen in sparse cells was almost entirely lost, leaving only a nuclear pattern. This was not seen in confluent cells. This effect was quantitated using a CCD camera. Thus, cells in sparse conditions contained VHL in both the nucleus and the cytosol, but cytosolic VHL could not be cross-linked to other cellular components. Cytosol VHL could be cross-linked in confluent cells. Elongin box VHL mutants could not be cross-linked and stabilized in the cytosol under any growth conditions, suggesting that the VBC interaction was essential for cytoplasmic cross-linking. Noting that under sparse conditions VHL-GFP could not be fixed in the cytosol led us to ask whether VHL was cycling between the nucleus and the cytosol. To test this, we used a variety of drugs hoping that one or more would block either nuclear import or nuclear export and result in a shift in the distribution of the VHL protein. Such an effect was seen with the transcriptional inhibitor actinomycin D. For VHL that cannot be cross-linked and stabilized in the cytosol, the addition of actinomycin D resulted in a rapid redistribution of VHL from cytosol to the nucleus. This was particularly rapid for mutant VHL that cannot bind Elongin B/C, but was also seen for wild-type VHL, although at a slower rate, and only under sparse conditions. In contrast, wild-type VHL in confluent cells shows no redistribution of VHL over two hours in the presence of actinomycin D. This effect could not be duplicated with inhibitors of protein synthesis. This suggested a model whereby VHL cycles between the cytosol and the nucleus requiring ongoing transcription in order to be exported from the nucleus. One step in the cycle that appears to be regulated by environmental signals, in this case confluency, is retention in the cytosol which can be measured both by the failure to redistribute the presence of actinomycin D and the ability to cross-link the VHL in the cytosol to undefined cellular components.

We sought to correlate the distribution and cycling of VHL with the regulation of VEGF mRNA levels. We asked whether cells expressing a mutant or wild-type VHL demonstrated altered VEGF mRNA as a function of confluency, under the same conditions used to observe the redistribution of the VHL protein. We found that in a variety of kidney cancer cell lines, VEGF mRNA levels rose dramatically when cells were grown in confluent conditions when compared to sparse conditions. This was only seen in cells failing to express VHL or expressing mutant VHL. In cells expressing wild-type VHL, VEGF mRNA levels did not change as a function of confluency. It appears from these results that confluency, independent of VHL, regulates VEGF mRNA levels. At the same time, confluency results in some alteration that affects the distribution of VHL resulting in it being stably in the cytoplasm interacting with other cellular components. This simultaneous affect on VHL distribution and VEGF mRNA by confluency cancel each other and wild-type VHL abrogates the ability to induce VEGF mRNA levels in response to confluency. These results strongly suggest that VEGF mRNA levels, which are regulated by VHL at the level of mRNA stability, requires VHL to be in the cytosol.

VHL Function in the Mouse

In order to determine the effect of heterozygous or homozygous deletion of VHL in a mammal, knock-out mice were generated by standard techniques. Heterozygous mice develop normally and out to one year we have seen no evidence of tumors. In contrast, homozygous knock-out of VHL is an embryonic lethal event. We have recently attempted to pinpoint the point in embryonic development at which this lethality is seen. It appears to be very early between embryonic day 7.5 and 8.5 and precedes organogenesis. The most striking abnormality is the failure to develop a structure called the labyrinth, a vascular structure elaborated by the embryo that allows the intermixing of maternal and fetal blood across endothelial barriers. In the absence of VHL, there is essentially no significant development of the vasculature in this organ and no fetal nucleated red cells present. This region of the fetus demonstrates hemorrhage resulting from the extrusion of maternal cells and presumably is responsible for the early and uniform loss of the embryos.

TRANSCRIPTIONAL REGULATION

A new group, headed by David Wassarman, has been formed to undertake a genetic analysis of RNA polymerase II transcriptional factor in *Drosophila*. Transcriptional regulation is a basic process that occurs in all eukaryotic organisms. Mutations that affect the level, timing, or spacial distribution of transcription can cause severe developmental abnormalities. Since the transcriptional machinery displays a high degree of evolutionary conservation, an understanding of the factors and mechanisms that regulate transcription in *Drosophila* should be easily extrapolated to humans.

Proposed Course of Project:

1. Genetic screens for transcription factors: Using sensitized genetic backgrounds that affect Drosophila eye development, we will conduct genetic screens to isolate mutations in known components of the RNA polymerase II transcriptional machinery, as well as identify general and gene-specific components of this multiprotein complex. The isolation of mutations in this class of genes will provide important tools for studying transcriptional regulation during Drosophila development.

2. Analysis of promoter utilization and recognition by TAFs: Mutations in TAFs suppress rough eye phenotypes caused by genes expressed under control of the sevenless (sev) enhancer and promoter but not the sev enhancer and heat shock protein 70 (hsp70) promoter. Using the rough eye phenotype as a reporter for transcription level in vivo, the sev, hsp70, and other promoters will be mutagenized and assayed for altered phenotypes in TAF mutant flies. In this manner, it may be possible to define promoter sequences and specific TAFs that are involved in promoter discrimination.

3. Characterization of sev transcription factors: A number of the screens described above rely on sev regulatory sequences to express genes in the Drosophila eye. To understand how TAFs are functioning in this context, it is important to identify factors and DNA sequences that regulate sev transcription. Previous studies have defined a 475-bp enhancer fragment that confers correct qualitative and quantitative expression of the sev gene. Enhancer binding factors that recognize this fragment and activate transcription will be identified by molecular methods. In addition, mutations in these genes may be isolated in the genetic screens.

4. Biochemical analysis of mutant transcription factors: Mutant proteins isolated in the screens described above will be assayed in vitro to determine how they affect transcriptional activation.

Bibliography

Boll W, Ohno H, Songyang Z, Rapoport I, Cantley LC, Bonifacino JS, Kirchhausen T. Minimal sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes. EMBO J. 1996; in press.

Bonifacino JS, Marks MS, Ohno H, Kirchhausen T. Mechanisms of signal-mediated protein sorting in the endocytic and secretory pathways. Proceedings of the Association of American Physicians 1996; 108, in press.

Bruggemann E, Handwerger K, Essex C, Storz G. Analysis of fast neutron-generated mutants at the *Arabidopsis thaliana HY4* locus. Plant J 1996;in press.

Butt J, Kim HY, Basilion JP, Cohen S, Iwai K, Philpott CC, Altschul S, Klausner RD, Rouault TA. Differences in the RNA binding sites of iron regulatory proteins and potential target diversity. PNAS 1996;93:4345-4349.

Cole NB, Sciaky N, Marotta A, Song J, Lippincott-Schwartz J. Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral ER exit sites. Mol Biol Cell 1996;7:631-650.

Cole NB, Smith C, Sciaky N, Terasaki M, Edidin M, Lippincott-Schwartz J. Diffusional mobility of Golgi proteins in membranes of living cells. Science 1996;273:797-801.

Delahunty MD, Bonifacino JS. Disorders of intracellular protein trafficking in human disease. Conn Tissue Res 1995;31:283-6.

Delahunty MD, Rhee I, Freed EO, Bonifacino JS. Mutational analysis of the fusion peptide of human immunodeficiency virus type 1: identification of critical glycine residues. Virology 1996; 218:94-102.

Donovan JA, Ota Y, Langdon WY, Samelson LE. Regulation of the association of $p120^{cbl}$ with Grb2 in Jurkat T cells. J Biol Chem 1996;in press.

Gnarra JR, Duan DR, Weng Y, Humphrey JS, Chen DYT, Lee S, Pause A, Dudley CF, Latif F, Kuzmin I, Schmidt L, Duh FM, Lerman MI, Zbar B, Klausner RD, Linehan WM. Molecular cloning of the von Hippel-Lindau tumor suppressor gene and its role in renal carcinoma. Biochim Biophys Acta 1996;1242:201-210.

Gnarra J, Zhou S, Merrill MJ, Wagner J, Krumm A, Papavassiliou E, Oldfield E, Klausner RD, Linehan WM. Post-transcriptional regulation of vascular endothelial growth factor mRNA by the VHL tumor suppressor gene product. PNAS 1996;in press.

Isakov N, Wange RL, Watts JD, Aebersold R, Samelson LE. Purification and characterization of human ZAP-70 from a baculovirus expression system. J Biol Chem 1996;271:15753-15761.

Iwai K, Klausner RD, Rouault TA. Requirements for iron regulated degradation of the RNA binding protein, Iron Regulatory Protein 2 (IRP2). EMBO J 1995;14:5350-5357.

Kim HY, LaVaute T, Iwai K, Klausner RD, Rouault TA. Identification of a conserved and functional iron-responsive element in the 5'UTR of mammalian mitochondrial aconitase. JBC 1996;in press.

Kolesnitchenko V, Wahl LM, Tian H, Sunila I, Tani Y, Hartmann D-P, Cossman J, Raffeld M, Orenstein J, Samelson LE, Cohen DI. HIV-1 envelope-initiated G2-phase programmed cell death. Proc Natl Acad Sci USA 1995;92:11889-11893.

Lee S, Chen DYT, Humphrey JS, Gnarra JR, Linehan WM, Klausner RD. Nuclear/cytoplasmic localization of the VHL tumor suppressor gene product is determined by cell density. PNAS 1996;93:1770-1775.

Lippincott-Schwartz J. Membrane traffic and compartmentalization within the secretory pathway. In: Schultz et al, eds. Molecular biology of membrane transport disorders. Plenum Press, 1996;1-9.

Marks MS, Roche P, van Donselaar E, Woodruff L, Peters PJ, Bonifacino JS. A lysosomal targeting signal in the cytoplasmic tail of the beta chain directs HLA-DM to the MHC class II antigen processing compartment. J Cell Biol 1995;131:351-269.

Marks MS, Woodruff L, Ohno H, Bonifacino JS. Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. J Cell Biol 1996; in press.

Muffler A, Fischer D, Altuvia S, Storz G, Hengge-Aronis R. The response regulator RssB controls stability of the σ^s subunit of RNA-polymerase in *Escherichia coli*. EMBO J 1996;15:1333-39.

Ohno H, Fournier MC, Poy G, Bonifacino JS. Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. J Biol Chem 1996; in press.

Ohno H, Kirchhausen T, Bonifacino JS. Recognition of tyrosine-based sorting signals by the medium chains of clathrin-associated protein complexes. J NIH Res 1995;7:50-1.

Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T and Bonifacino JS. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 1995;269:1872-5.

Ooi CE, Rabinovich E, Dancis A, Bonifacino JS, Klausner RD. Copper-dependent degradation of the *S. cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. EMBO J 1996;15:3515-3523.

Ooi CE, Rabinovich E, Dancis A, Bonifacino JS, Klausner RD. Copper-dependent degradation of the S. cerevisiae plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. EMBO J 1996;15:3515-23.

Pause A, Aso T, Linehan WM, Conaway JW, Conaway RC, Klausner RD. Analysis of the interaction of the VHL tumor suppressor gene product with elongin. Methods in Enzymology 1996;in press.

Radhakrishna H, Klausner RD, Donaldson JG. Aluminum fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. JCB 1996;134:1-13.

Rouault TA, Klausner RD. The impact of oxidative stress on eukaryotic iron metabolism. In: Feige et al., eds. Stress-inducible cellular responses. Birkhäuser/Springer, 1996; in press.

Rouault TA, Klausner RD. Iron-sulfur clusters as biosensors of oxidants and iron. TIBS 1996;21:174-177.

Rouault TA, Klausner RD, Harford JB. Translational control of ferritin. In: Hershey J et al., eds. Translational regulation. Cold Spring Harbor: Cold Spring Harbor Press, 1996;335-362.

Rouault TA, Klausner RD. Regulation of iron metabolism in eukaryotes. In: Stadtman et al., eds. Current Topics in Cellular Regulation. Vol. 35. Academic Press, 1996; in press.

Rouault TA, Klausner RD. Post-transcriptional regulation of genes of iron metabolism in mammalian cells. J Bioinorganic Chem 1996; in press.

Shapiro J, Sciaky N, Lee J, Bosshart H, Angeletti R, Bonifacino JS. Localization of endogenous furin to the Golgi complex. J Histochem Cytochem 1996; in press.

Shatwell KP, Dancis A, Cross AR, Klausner RD, Segal AW. The FRE1 ferric reductase of *Saccharomyces cerevisiae* is a cytochrome *b* similar to that of NADPH oxidase. JBC 1996;271:14240-14244.

Stearman R, Yuan DS, Yamaguchi-Iwai Y, Klausner RD, Dancis A. A permease-oxidative complex required for high-affinity iron uptake in yeast. Science 1996;271:1552-1557.

Samelson LE, Donovan JA, Isakov N, Ota Y, Wange RL. Signal transduction mediated by the T cell antigen receptor. Annals of the NY Acad Sci 1995;766:157-172.

Shirley BW, Kubas WL, Storz G, Bruggeman E, Koornneef M, Ausubel FM, Goodman HM. Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. Plant J 1995;8:659-671.

Tian H, King L, Lempicki R, King L, Donoghue E, Samelson LE, Cohen DI. HIV-envelope-directed signalling aberrancies and cell death of CD4+ T cells in the absence of TCR co-stimulation. Int Immunol 1996;8:65-74.

Voorhees P, Diegnan E, van Donselaar E, Humphrey J, Marks MS, Peters PJ, Bonifacino JS. An acidic sequence within the cytoplasmic domain of the furin functions as a determinant of TGN localization and internalization from the cell surface. EMBO J 1995;14:4961-4975.

Wange RL, Samelson LE. Complex complexes: signaling via the TCR. Immunity 1996;in press.

Watts JD, Affolter M, Krebs DL, Wange RL, Samelson LE, Aebersold R. Electrospray ionization mass spectrometric investigation of signal transduction pathways: determination of sites of inducible protein phosphorylation in activated T cells. In: Snyder AP, ed. Biochemical and biotechnological applications of electrospray ionization mass spectrometry. American Chemical Society, 1996; 381-407.

Wienecke R, Maize Jr JC, Shoarinejad F, Vass WC, Reed J, Bonifacino JS, Resau JH, de Gunzburg J, Yeung RS, DeClue JE. Co-localization of the TSC2 product tuberin with its target Rap1 in the Golgi apparatus. Oncogene 1996;in press.

Yamaguchi-Iwai Y, Stearman R, Dancis A, Klausner RD. Iron-regulated DNA binding by the AFT1 protein controls the iron regulation in yeast. EMBO J 1996;15:3377-3384.

CELL BIOLOGY AND METABOLISM BRANCH (FY96)

Chief, Richard D. Klausner, M.D.

Principal Investigators

Juan S. Bonifacino, Ph.D.
Tracey A. Rouault, M.D.
Lawrence E. Samelson, M.D.

Tenure-Track Investigators

Jennifer Lippincott-Schwartz, Ph.D.
Gisela Storz, Ph.D.
David Wassarman, Ph.D

Senior Fellows

Andrew Dancis, M.D.
Daniel Yuan, M.D.

Post-Doctoral Fellows

James Basilion, Ph.D.
Edward Bruggeman, Ph.D.
Martha Delahunty, Ph.D.
Esteban Dell'Angelica, Ph.D.
Jerald Donovan, Ph.D., M.D.
Alan Finegold, Ph.D.
Frans Hochstenbach, Ph.D.
Jeffrey Humphrey, M.D.
Noah Isakov, Ph.D.
Kazuhiro Iwai, M.D., Ph.D.
Yuko Iwai, Ph.D.
Hae Yeong Kim, Ph.D.
Tiit Land, Ph.D.
Stephen Lee, Ph.D.
Michael Marks, Ph.D.
Maria Merlotti, Ph.D.
Hiroshi Ohno, M.D., Ph.D.
Chean Ooi, Ph.D.
Yasuo Ota, M.D.
Arnim Pause, Ph.D.
John Presley, Ph.D.
Joanne Sloan-Lancaster, Ph.D.
Wing-Hang Tong, Ph.D.
Ronald L. Wange, Ph.D.
Kristien Zaal, Ph.D.
Aixia Zhang, Ph.D.

Weiguo Zhang, Ph.D.
Ming Zheng, M.D.

Pre-Doctoral Fellows
Bruce Brown
Nelson Cole
Jan Ellenberg
Marie Christine Fournier
Korie Handwerger
Andrew Hoffnagle
Jason Kitchen
Quang Phan
Virginia Su

Guest Researchers
Herbert Bosshart, M.D.
David Chen
David Cohen, M.D.
Patricia DeRusso, M.D.
Victor Gordeuk, M.D.
Sandra Hasstedt
Vincent Kolesnitchenko, Ph.D.
Peter Peters, Ph.D.
Efrat Rabinovich

Senior Technical Specialists
Timothy LaVaute, Ph.D.
Robert Stearman, Ph.D.

Project Numbers - Annual Report 1996

CELL BIOLOGY AND METABOLISM BRANCH (CBMB)

ZO1 HD 01600-12	Biochemical Basis of T Cell Activation Larry E. Samelson, M.D.
ZO1 HD 01602-12	Regulation of Intracellular Iron Metabolism Tracey A. Rouault, M.D.
ZO1 HD 01606-07 **TERMINATED**	The Biology of Early Organelles of the Secretory Pathway Richard D. Klausner, M.D.
ZO1 HD 01607-06	Protein Trafficking in the Secretory Pathway Juan Bonifacino, Ph.D.
Z01 HD 01608-06	Gene Regulation in Response to Environmental Stress Gisela Storz, Ph.D.
Z01 HD 01609-05	Localization and Dynamics of Intracellular Organelles Jennifer Lippincott-Schwartz, Ph.D.
Z01 HD 01610-04	Intracellular Metal Metabolism Richard D. Klausner, M.D.
Z01 HD 01611-02	The VHL Tumor Suppressor Gene Richard D. Klausner, M.D.
Z01 HD 01612-01	Genetic Analysis of RNA Polymerase II Transcriptional Regulation in Drosophila David A. Wassarman, Ph.D.

Z01 HD 01613-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-HD-01600-12

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Basis of T Cell Activation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.E. Samelson	Chief , SLS	CBMB, NICHD			
Others:	J. Donovan	IRTA	CBMB, NICHD	R.L. Wange	IRTA	CBMB, NICHD
	N. Isakov	Adjunct Scientist	CBMB, NICHD	W. Zhang	SUP-IRTA	CBMB, NICHD
	J. Kitchen	Pre-IRTA	CBMB, NICHD			
	Y. Ota	Visiting Fellow	CBMB, NICHD			
	J. Sloan-Lancaster	SUP-IRTA	CBMB, NICHD			
	V. Su	Pre-IRTA	CBMB, NICHD			

COOPERATING UNITS *(if any)*

Dept. Biotech., U. WA (R. Aebersold and J. Watts); Dept. Biochem., U. of W. Australia (W. Langdon); Dept. Path., Beth Israel Hosp., Harvard Med.Schl. (J.P. Kinet); Dept.Immun., Mayo Clinic (R. Abraham)

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Section on Lymphocyte Signaling

INSTITUTE AND LOCATION

NICHD, NIH

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.0	6.4	.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Engagement of the multicomponent antigen receptor in T cells (TCR) results in rapid activation of a protein tyrosine kinase pathway. A major TCR- associated protein tyrosine kinase is ZAP-70, a protein that binds to the activated TCR. Under conditions of TCR activation, the ZAP-70 bound to the TCR is itself tyrosine phosphorylated and activated. We have expressed this enzyme using a baculovirus system and have developed a purification scheme enabling us to obtain sufficient ZAP-70 for enzymatic analysis. Our studies of the mechanism of ZAP-70 activation includes further analysis of mutants of the enzyme that lack critical tyrosine residues. Expression of these mutants in T cells that lack endogenous ZAP-70 leads to the conclusion that phosphorylation of this enzyme is critical to its regulation. Additional studies focus on a substrate of tyrosine kinases in T cells, $p120^{cbl}$, a proto-oncogene, which may be involved in the Ras pathway in T cells. Earlier studies demonstrate that this protein can be found in a complex with the linker protein Grb2. We have mapped the interaction and have demonstrated that the Grb2-$p120^{cbl}$ complex is regulated by TCR activation. Engagement of the Fc receptor for IgE on mast cells also results in $p120^{cb}$ tyrosine phosphorylation. In these cells we have demonstrated that the Syk protein tyrosine kinase is primarily responsible for this phosphorylation. Moreover, Syk and $p120^{cbl}$ can be found in a complex whose function is under investigation.

BIOCHEMICAL BASIS OF T CELL ACTIVATION

Project Description

Objectives:

The T cell antigen receptor (TCR), like the B cell antigen receptor and receptor for Ig (Fc receptors), is a multicomponent structure that is coupled to several signal transduction pathways. Our earlier studies have demonstrated that receptor occupancy results in activation of tyrosine and serine kinases. Our current focus is the complete characterization of the protein tyrosine kinase pathway. The major goal of the unit is to identify and characterize the responsible kinase(s) and major substrates of these kinases. Only by this means will we be able to understand the function of this pathway and the manner by which it is activated and regulated.

Methods Employed:

Our studies of T cell activation use antigen specific murine T cell hybridomas, human T cell tumors, normal human peripheral T cells and murine thymocytes. A similar receptor system, the receptor for IgE on mast cells has also been analyzed using tumor lines. Activation is induced either by specific antigen or stimulatory monoclonal antibodies. Analysis of signal transduction mechanisms depends on metabolic radiolabeling, detergent solubilization, immunoprecipitation and polyacrylamide gel electrophoresis. Phosphopeptide mapping has been performed to define sites of protein phosphorylation. Immunoblotting is used to detect antigen receptor subunits and tyrosine phosphorylated proteins. Immune complex kinase assays are used to determine protein tyrosine kinase activity in vitro. Protein tyrosine kinase domains are expressed in bacteria in order to obtain sufficient material for binding studies. Kinase protein is prepared using baculovirus expression systems. Phosphopeptides of defined sequence are synthesized to order. Kinase substrates have been purified by immunoaffinity chromatography, amino acid sequence has been obtained, and the cDNA encoding a protein tyrosine kinase substrate has been cloned. Mutated forms of this substrate have been generated using standard site-directed techniques. Eukaryotic and vaccinia vectors have been used to enable expression of proteins in lymphoid cells. Flow cytometry is used to characterized cell cycle progression.

The T Cell Receptor Tyrosine Kinase(s):

Identifying the kinase(s) responsible for tyrosine phosphorylation of the TCR and the other cellular substrates has been a major goal of this Section for many years. A critical tyrosine kinase activated by the T cell receptor is the ZAP-70 protein, which is tyrosine phosphorylated and associates with the TCR upon activation. In the past year we developed reagents and methods enabling us to obtain large quantities of pure ZAP-70 for analysis. Recombinant human ZAP-70 was overexpressed in insects cells after infection with a ZAP-70-containing baculovirus. Detergent-soluble extracts were subjected to dye affinity resins and sizing columns. Analysis by SDS-PAGE revealed one 70kD band, which was reactive with ZAP-70 specific reagents. The enzyme in this preparation is active. It was used to demonstrate a requirement for Mn^{++} and a pH optimum of 6.5. Using the cytoplasmic fragment of band 3 (cfb3) as an in vitro substrate, we demonstrated an apparent K_m for ATP of 3.0μM. Kinetic values were also obtained for substrate phosphorylation. We also found that ZAP-70, unlike Lck, had a very restricted capacity to phosphorylate proteins in vitro. Only cfb3 and tubulin were phosphorylated by active ZAP-70, whereas many more examples of substrates for Lck were demonstrated. We also tested the ability of ZAP-70 to phosphorylate the tyrosine residues found in the tails of TCR subunits. These tyrosines, after phosphorylation by Lck or Fyn serve as binding sites for the SH2 domains of ZAP-70. In vitro we confirmed that recombinant Lck could phosphorylate peptides with these sites. However, ZAP-70 was unable to catalyze phosphorylations of these tyrosines. In a final experiment for this study we asked whether phosphopeptide binding to ZAP-70 would lead to enzymatic activation. It has been suggested that such binding would serve as an allosteric activation event for this enzyme. We were unable to demonstrate this effect. As described below, all evidence suggests that ZAP-70 is regulated by phosphorylation.

With collaborators in Vancouver and Seattle we previously mapped the sites of tyrosine phosphorylation of the ZAP-70 kinase. Residues Y292, Y492 and Y493 become phosphorylated after TCR engagement. These sites were then analyzed by site directed mutagenesis and expression in COS cells. In this earlier study we demonstrated that Y493 was absolutely required for kinase activation, most likely because it serves as the site of Lck phosphorylation. The mutation of Y492 to phenylalanine gave the unexpected result of leading to a four-fold increase in kinase activity. We saw no effect of a Y to F mutation at Y292 after testing in COS cells. More recently we have obtained a mutant form of the Jurkat T cell line that lacks ZAP-70. TCR engagement of this cell fails to lead to cellular activation, as there is minimal induction of tyrosine phosphorylation, no calcium flux and no lymphokine production observed. Reconstitution of the cells with ZAP-70 results in restoration of TCR-coupled signaling. Reconstitution with the various Y to F mutants was then performed. As expected the Y493F mutation failed to signal in the T cells. The Y492F and the Y292F versions of ZAP-70 both led to prolonged activation of tyrosine phosphorylation and enhanced lymphokine production as measured by IL-2 promoter transcriptional activation. These data confirm that ZAP-70 function is regulated by its tyrosine phosphorylation. One likely explanation of the significance of the phosphorylation at Y292 and Y492 is that they serve as binding sites for proteins that regulate function.

The Tyrosine Phosphorylation Pathway in T cells:

In previous studies we have demonstrated that activation of the TCR results in phosphorylation of the TCRζ chain and multiple intracellular substrates. The identity of the other substrates that are tyrosine phosphorylated upon TCR activation has been a major question for this Section for some time. One substrate of TCR-mediated tyrosine kinase activation is the protooncogene p120^{cbl}, which is rapidly tyrosine phosphorylated upon T cell activation. We showed earlier that the SH3 domains of the linker molecule Grb2 binds to p120^{cbl} in vitro. More recently we have demonstrated that a Grb2-p120^{cbl} complex can be found in the cell. The complex dissociates upon TCR engagement with kinetics suggesting that the tyrosine phosphorylation of p120^{cbl} induces the dissociation. However more extensive analysis suggests that additional factors are responsible, since dephosphorylation does not induce reassociation. The complex of Grb2 and $p120^{cbl}$ was further studied by mapping the interaction of the proteins. $p120^{cbl}$ contains a 200 amino acid region with multiple potential SH3 binding sites. Our analysis demonstrates that a construct (1-528) containing the sequence PPVPPR binds Grb2. However increased binding can be detected in longer constructs containing additional proline-rich sequences. Multiple proline rich sites could serve as alternate sites of interaction or aid in generating a structure better able to interact with SH3 domains.

The function of $p120^{cbl}$ was also studied in RBL-2H3 cells which can be activated by engagement of the FcεRI. We demonstrated that in this system $p120^{cbl}$ is also tyrosine phosphorylated upon receptor engagement. Using recombinant vaccinia viruses to overexpress various protein tyrosine kinases, we demonstrated that Syk, a kinase closely related to ZAP-70 is responsible for $p120^{cbl}$ tyrosine phosphorylation in RBL-2H3 cells. In addition we mapped the site of $p120^{cbl}$ tyrosine phosphorylation to the distal end of the molecule. We found that $p120^{cbl}$ and Syk form a complex in RBL-2H3 cells. This too was analyzed by overexpression. The tandem SH2 domain of Syk was sufficient to bind residues 1-655 of $p120^{cbl}$. Interestingly we also found that the $p120^{cbl}$ found within this complex was not tyrosine phosphorylated. This result suggests that there may be functional significance to the interaction of $p120^{cbl}$ with the Syk kinase.

Publications

Donovan JA, Ota Y, Langdon WY, Samelson LE. Regulation of the association of $p120^{cbl}$ with Grb2 in Jurkat T cells. J Biol Chem 1996;in press.

Isakov N, Wange RL, Watts JD, Aebersold R, Samelson LE. Purification and characterization of human ZAP-70 from a baculovirus expression system. J Biol Chem 1996;271:15753-15761.

Kolesnitchenko V, Wahl LM, Tian H, Sunila I, Tani Y, Hartmann D-P, Cossman J, Raffeld M, Orenstein J, Samelson LE, Cohen DI. HIV-1 envelope-initiated G2-phase programmed cell death. Proc Natl Acad Sci USA 1995;92:11889-11893.

Samelson LE, Donovan JA, Isakov N, Ota Y, Wange RL. Signal transduction mediated by the T cell antigen receptor. Annals of the NY Acad Sci 1995;766:157-172.

Tian H, King L, Lempicki R, King L, Donoghue E, Samelson LE, Cohen DI. HIV-envelope-directed signalling aberrancies and cell death of CD4+ T cells in the absence of TCR co-stimulation. Int Immunol 1996;8:65-74.

Wange RL, Samelson LE. Complex complexes: signaling via the TCR. Immunity 1996;in press.

Watts JD, Affolter M, Krebs DL, Wange RL, Samelson LE, Aebersold R. Electrospray ionization mass spectrometric investigation of signal transduction pathways: determination of sites of inducible protein phosphorylation in activated T cells. In: Snyder AP, ed. Biochemical and biotechnological applications of electrospray ionization mass spectrometry. American Chemical Society, 1996;381-407.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-HD-01602-12

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Intracellular Iron Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T.A. Rouault	Chief, SHIM	CBMB, NICHD	Co-PI:	R.D. Klausner	Chief, CBMB	
Others:	J. Basilion	Staff Fellow	CBMB, NICHD		T. LaVaute	Biologist	CBMB, NICHD
	P. DeRusso	Adjunct Scientist	CBMB, NICHD		C. Philpott	IRTA	CBMB, NICHD
	A. Hoofnagle	Pre-IRTA	CBMB,NICHD		W.H. Tong	Adjunct Scientist	CBMB, NICHD
	K. Iwai	Adjunct Scientist	CBMB, NICHD				
	H.-Y. Kim	Adjunct Scientist	CBMB, NICHD				
	T. Land	Adjunct Scientist	CBMB, NICHD				

COOPERATING UNITS *(if any)*

Scripps Research Institute (D.Stout); American Red Cross (W. Burgess);, University of Colorado (A. Pardi);, University of Wisconsin (H. Beinert & C. Kennedy); NHLBI (R. Levine).

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Section on Human Iron Metabolism

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
8.80	8.	.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project is aimed at understanding the molecular basis of intracellular iron metabolism. The cis and trans elements mediating the iron-dependent alterations in abundance of ferritin and the transferrin receptor have been identified and characterized in previous years in this laboratory. Iron-responsive elements (IREs) are RNA stem-loops found in the 5' end of ferritin mRNA and the 3' end of transferrin receptor mRNA. We have cloned, expressed, and characterized two essential iron-sensing proteins, Iron Regulatory Protein 1 (IRP1) and Iron Regulatory Protein 2 (IRP2), formerly referred to as iron-responsive element binding proteins (IRE-BPs). IRPs bind IRE's when iron levels are depleted, resulting in the inhibition of translation of ferritin mRNA and prolongation of the half-life of the transferrin receptor mRNA. IRP1 is an iron-sulfur protein related to mitochondrial aconitase, a Krebs cycle enzyme, and it functions as a cytosolic aconitase in cells that are iron replete. Regulation of RNA binding activity of IRP1 involves a transition from a form of IRP1 in which a [4Fe-4S] cluster is bound, to a form that loses both iron and aconitase activity. The [4Fe-4S] containing protein does not bind IREs. Controlled degradation of the iron-sulfur cluster reveals that the physiologically relevant form of the RNA binding protein in iron-depleted cells is apoprotein. The status of the cluster appears to be the key to determining whether the protein will bind RNA. Recently, we have identified mammalian genes that are homologous to the NifS and Nif U genes that are implicated in bacterial iron-sulfur cluster assembly, and we have shown that these gene products facilitate assembly of the iron-sulfur cluster of IRP1. Unlike IRP1, IRP2 is rapidly degraded in cells that are iron-replete. An additional exon present in IRP2 determines whether IRP2 is subject to degradation. This 73 amino acid exon is sufficient to confer upon IRP1 an iron -dependent degradation phenotype. Mutagenesis of cysteines within the 73 amino acid domain reveals that cysteines are required for degradation. We have recently shown that IRP2 is oxidatively modified in iron-replete cells, and that the modified intermediate is ubiquitinated prior to being degraded by the proteasome. In order to approach questions about the physiology of iron metabolism, IRP1 has been "knocked out" in mice, using homologous recombination in embryonic cell lines. There is no obvious phenotype associated with loss of IRP1 function, and we speculate that both IRPs must be absent in order to see perturbations in iron metabolism. Accordingly, creation of IRP2 "knockouts" and double "knockouts" is underway.

PHS 6040 (Rev. 5/92)

REGULATION OF INTRACELLULAR IRON

Project description:

Objectives:

The main objective of this project is to understand mammalian iron metabolism. We have cloned and characterized through site-directed mutagenesis the two major regulatory proteins involved in sensing iron levels, IRP1 and IRP2. In order to better understand their mode of regulation, iron-sulfur cluster assembly and disassembly is being studied. Enzymes of iron-sulfur cluster assembly have been cloned. Pulse-chase studies have been used to define the iron-dependent degradation of IRP2 and mutagenesis is being utilized to better characterize the iron-dependent degradation signals. Over-expressed and purified apoprotein is being used to attempt to create crystals of the apoprotein and co-crystals with the RNA ligand. Homologous recombination and electroporation of embryonic stem cells has been used to created mice that are heterozygous or homozygous for deficiency of IRP1 and/or IRP2.

Methods employed:

Standard techniques of molecular biology are employed including cloning, site-directed mutagenesis using 2 step PCR methodology, and transient and stable transfection. Protein chemistry techniques include one and two-dimensional electrophoresis, immunoprecipitation, biosynthetic labeling, and FPLC. Gel-retardation and resolution of complexes is performed on native gels, and in vitro translation systems are employed.

Major findings:

Cloning and characterization of enzymes involved in assembly of iron-sulfur clusters in cytosol and mitochondria of eukaryotic cells

Enzymes homologous to enzymes involved in assembly of iron-sulfur clusters of bacteria have been cloned in yeast and in mammalian cells. The homologues of bacterial NifS and NifU genes are localized to the mitochondria or cytosol of cells using specific 5' targeting signals. These gene products appear to coassemble with several other proteins to facilitate insertion of iron-sulfur clusters into target proteins. Yeast genetics and biochemical techniques are being used to fully characterize the molecular complex and to determine the mechanism of assembly of iron-sulfur clusters.

IRP2 contains a transferrable iron-dependent degradation domain

Binding studies have shown that IRP2 binds to consensus IREs with high affinity and specificity similar to IRP1. Whereas the RNA binding activity of IRP1 is regulated by the reversible assembly and disassembly of an iron-sulfur cluster in an otherwise stable protein, absolute levels of IRP2 are markedly decreased by iron treatment in several different cell types and the decrease in protein levels can be accounted for by an increase in the rate of degradation of IRP2. IRP1 and IRP2 are highly homologous proteins with an overall sequence identity of 58%. A major distinguishing feature between IRP1 and IRP2 is the presence of an insertion of 73 amino acids in domain 1 of IRP2. Cloning of the genomic fragment corresponding to this sequence has revealed that the 73 amino acids are encoded by a single exon. When the 73 amino acid insertion was excised by site directed mutagenesis from IRP2, levels of the IRP2 exon deletion mutant (IRP2-73) were no longer markedly decreased after treatment with iron. To assess whether the 73 amino acid IRP2-specific exon contained sufficient information to direct iron regulated degradation of IRP2, the sequence was cloned into IRP1 at a homologous position. When assayed in a pulse chase experiment in the presence of iron, the IRP1/IRP2 specific exon chimeric protein (IRP1+73) was much more rapidly degraded than wild-type IRP1 and the degradation rate was comparable to that of wild type IRP2 (t1/2 of approximately 3-6 h). Thus, the IRP2 specific exon conferred the capacity to be degraded at a rate similar to that seen in intact IRP2 upon IRP1. In order to test whether cysteines from the IRP2 specific exon were important in degradation, the degradation rate of the chimeric protein IRP1/IRP2 specific exon (IRP1+73) was compared to the degradation rate of a similar construct which differed only in that the middle three cysteines, C168, C174 and C178, of the IRP2 specific domain were simultaneously converted to serines by site-directed mutagenesis (Exon 3C-S). When the three cysteines were mutated to serines, the IRP2 specific exon could no longer transfer the capacity for rapid iron-dependent degradation to IRP1. A pulse chase experiment revealed that the exon 3C-S mutant was stable, with an estimated t1/2 of 24 h. In order to gain insight into the mechanism of degradation, iron-replete cells were treated with a variety of reagents known to interfere with various modes of proteolysis in cells. The peptide aldehyde MG132, a potent inhibitor of the 20 S subunit of the proteasome, interfered with degradation of IRP2 when cells were treated with iron. To further confirm the role of the proteasome in degradation of IRP2, a more specific inhibitor of proteasome function, lactacystin, was used. Lactacystin inhibits proteasome function by covalently binding to the amino terminal threonine of the mammalian proteasome subunit X. The effects of lactacystin in the cell are more specific for the proteasome than are those of MG132 and we found that lactacystin inhibits degradation of IRP2 at a level comparable to MG132 (Iwai et al., 1995). Use of proteasome inhibitors has permitted us to characterize intermediates in the degradation pathway. We have determined that IRP2 that is destined to be degraded is oxidatively modified as indicated by acquisition of carbonyl groups, and that the marked protein is selectively ubiquitinated.

Differences in the RNA binding sites of IRP1 and IRP2 may be important in potential target diversity

Using purified IRP1 and IRP2, we have performed a limited SELEX procedure and determined that there is an alternative RNA stem-loop ligand that binds to IRP1 with high affinity; the sequence of the loop is UAGUAX instead of CAGUGX. A different alternative ligand for IRP2 consists of the sequence GGGAGX. Mutagenesis of arginines important in IRE binding revealed that arginines of the active site cleft that were identified as important in the IRE binding site of IRP1 are also important in the IRE binding site of IRP2, but the pattern of dependence on each arginine differs. These data indicate that the RNA binding sites of the two IRPs differ in ways that may prove to be important. It is possible that some endogenous ligands are bound specifically by one, but not the other of the two IRPs.

Other transcripts that are regulated by IRP1 and IRP2

We have recently shown that the transcript of mitochondrial aconitase contains a functional IRE. However, the range of regulation is less than that seen with the ferritin IRE, and the levels of mitochondrial aconitase are decreased in animals on a low iron diet by only two-fold. The IRE of mitochondrial aconitase is less stable, and the IRE conformation may only be intermittently present, possibly accounting for the smaller degree of translational repression seen.

The role of IRPs in physiology

Homologous recombination has been used to "knockout" IRP1 in mice. Heterozygotes for the "knockout of IRP2 have been created. Mice lacking IRP1 only are healthy, and iron uptake and metabolism appear to be normal. However, when cells of the knockout mice are treated with hydrogen peroxide, the cells lacking IRP1 do not repress ferritin biosynthesis, whereas cells containing IRP1 do repress synthesis, most likely because of disassembly of the iron-sulfur cluster of IRP1. Breeding of the mice should enable us to assess the phenotype of mice lacking the gene products of both IRP1 and IRP2.

Proposed course of the project

1. We plan to clone and characterize the iron-sulfur cluster assembly complex in cytosol and in mitochondria in both mammalian cells and yeast. We plan to use coprecipitation methodologies to identify all the components of the complex and we will determine the function of each protein in contributing to iron-sulfur cluster assembly. Our preliminary observations indicate that the mitochondrial and cytosolic forms of NifS are derived from a single transcript in which alternative usage of in-frame AUGs determines whether a targeting sequence will be present. We will study how distribution of translation products is regulated. Using yeast genetics, we will study targeting and assembly of iron-sulfur clusters in the mitochondria, making use of the ability of yeast to tolerate respiratory insufficiency.

2.We plan to use biochemistry and two-hybrid technology to isolate the ubiquitination enzyme(s) responsible for ubiquitination of the oxidized form of IRP2. We will analyze the degradation domain more fully through mutagenesis and transfer the degradation domain to other proteins in an attempt to more completely understand the process of iron-dependent degradation.

3. Using mutagenesis, we will attempt to define other residues that are important in high affinity binding of IREs. We will use the NMR structure determined by Dr. Art Pardi and colleagues to model the IRE- IRP interaction and we will test our predictions with standard mutagenesis.

4. We will use purified IRP1 and IRP2 to isolate other potential ligands. Purified transcripts will be used to synthesize cDNA and products will be cloned and sequenced.

5. We will aim to create mice lacking in both IRP1 and IRP2 and will attempt to determine the role of these gene products in the physiology of iron uptake and distribution.

Significance to Biomedical Research and the Program of the Institute

Our work contributes to greater understanding of the uptake, utilization, and regulation of iron distribution, processes that are important in human nutrition. Regulation of iron uptake and distribution is important in cells and in animals because iron is indispensable in the function of numerous proteins, but excess iron is highly toxic. The reaction of iron with oxygen products creates free radicals and oxidative damage, and these reactions are thought to be important in processes such as aging and carcinogenesis. We are working towards identifying the quality control mechanisms used by cells in the defense against oxidative damage catalyzed by iron, and insight into mechanisms of iron-catalyzed oxidative damage could be important in improving the health of higher organisms.

Publications

Butt J, Kim HY, Basilion JP, Cohen S, Iwai K, Philpott CC, Altschul S, Klausner RD, Rouault TA. Differences in the RNA binding sites of iron regulatory proteins and potential target diversity. PNAS 1996;93:4345-4349.

Iwai K, Klausner RD, Rouault TA. Requirements for iron regulated degradation of the RNA binding protein, Iron Regulatory Protein 2 (IRP2). EMBO J 1995;14:5350-5357.

Kim HY, LaVaute T, Iwai K, Klausner RD, Rouault TA. Identification of a conserved and functional iron-responsive element in the 5'UTR of mammalian mitochondrial aconitase. JBC 1996;in press.

Rouault TA, Klausner RD. The impact of oxidative stress on eukaryotic iron metabolism. In: Feige et al., eds. Stress-inducible cellular responses. Birkhäuser/Springer, 1996;in press.

Rouault TA, Klausner RD. Iron-sulfur clusters as biosensors of oxidants and iron. TIBS 1996 21:174-177.

Rouault TA, Klausner RD, Harford JB. Translational control of ferritin. In: Hershey J et al., eds. Translational regulation. Cold Spring Harbor: Cold Spring Harbor Press, 1996;335-362.

Rouault TA, Klausner RD. Regulation of iron metabolism in eukaryotes. In: Stadtman et al., eds. Current Topics in Cellular Regulation. Vol. 35. Academic Press, 1996;in press.

Rouault TA, Klausner RD. Post-transcriptional regulation of genes of iron metabolism in mammalian cells. J Bioinorganic Chem 1996;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-HD-01606-07

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Biology of Early Organelles of the Secretory Pathway

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.D. Klausner	Chief, CBMB	
Others:	J. Donaldson	Senior Staff Fellow	CBMB, NICHD
	F. Hochstenbach	Visiting Fellow	CBMB, NICHD
	E. Rabinovich	Guest Researcher	CBMB, NICHD
	H. Radhakrisha	IRTA	CBMB, NICHD

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Section on Organelle Receptor Structure and Function

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

PROJECT HAS BEEN TERMINATED.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01607-06

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Protein Trafficking in the Secretory Pathway

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.S. Bonifacino	Visiting Scientist	CBMB, NICHD			
Others:	H. Bosshart	Guest Researcher	CBMB, NICHD	K. Roche	IRTA	CBMB, NICHD
	M. Delahunty	IRTA	CBMB, NICHD			
	E. Dell'Angelica	Visiting Fellow	CBMB, NICHD			
	M.-C. Fournier	Visiting Associate	CBMB, NICHD			
	H. Ohno	Visiting Fellow	CBMB, NICHD			
	C. Ooi	NRSA Fellow	CBMB, NICHD			

COOPERATING UNITS *(if any)*

U.Utrecht, The Netherlands (Peter Peters, Willem Stoorvogel); Harvard U. (Tomas Kirchhausen, Lewis Cantley), NCI (Allan Weissman), NIAID (Eric Freed), Yale U. (Ira Mellman)

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Unit on Intracellular Protein Trafficking, Sctn on Organelle Receptor & Function

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.3	5.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This group investigates the molecular mechanisms that determine the intracellular localization and sorting of integral membrane proteins in the endocytic and secretory pathways. This past year, our efforts have been primarily directed towards identifying and characterizing proteins that recognize cytoplasmic sorting signals. We had previously demonstrated that tyrosine-based sorting signals that mediate internalization from the cell surface and lysosomal targeting interacted specifically with the medium chains, μ_1 and μ_2, of the clathrin-associated adaptor complexes, AP-1 and AP-2. We have now conducted a detailed characterization of the factors that determine the specificity and the avidity of the signal-adaptor interactions. The requirements for interaction gleaned from these studies provide a mechanistic explanation for the involvement of tyrosine-based signals in various sorting processes. During the past year, we have also described the existence of a novel, ubiquitously-expressed adaptor-like complex that is, at least partially, associated with endosomes. This complex also recognizes tyrosine-based sorting signals and may be involved in sorting proteins for transport between early and late endosomes. Finally, we have collaborated with Allan Weissman (NCI) to identify the determinants of localization of the protein UBC6 to the endoplasmic reticulum (ER). The results of this study indicate that information leading to ER localization of UBC6 is contained within the membrane anchor of the protein and that the mechanism of localization may involve partitioning into membranes of different lipid composition.

PROTEIN TRAFFICKING IN THE SECRETORY PATHWAY

Project Description

Objectives:

This group investigates the molecular mechanisms that determine the localization and sorting of integral membrane proteins within the endocytic and secretory pathways. The main objectives of this project are the identification of signals involved in protein localization and sorting within the endosomal/lysosomal system and of recognition molecules that bind to the signals. A related objective is to understand the function of organellar coats in intracellular protein trafficking.

Methods Employed

All projects in this group involve the use of recombinant DNA procedures, morphologic and biochemical techniques, and yeast genetic approaches. In order to search for sorting signals within several integral membrane proteins, we constructed mutant and chimeric proteins using the polymerase chain reaction (PCR) and other standard recombinant DNA procedures. Constructs were expressed in mammalian cells by either transient or stable transfection. The subcellular localization of the different protein constructs was determined by immunofluorescence microscopy, image analysis, and quantitative immunoelectron microscopy, and their biosynthesis, processing and degradation were studied by pulse-chase metabolic labeling and immunoprecipitation. The ability of signals to mediate internalization from the cell surface was examined using quantitative endocytosis assays. Proteins that bind to sorting signals (recognition molecules) were studied using a yeast two-hybrid system and affinity purification with glutathione-S-transferase (GST) fusion proteins. The interaction of sorting signals with their recognition molecules was characterized using various *in vitro* binding assays, including surface plasmon resonance spectroscopy. Complementary DNAs encoding novel adaptor subunits were cloned using established recombinant DNA procedures. The expression, structure, and localization of novel adaptor-like complexes were studied using specific antibody reagents. Studies on the effect of overexpression of tyrosine-based sorting signals on protein sorting were performed by fluorescence activated cell sorting (FACS).

Major Findings

Introduction

The interior of the cell is organized into a diverse array of membrane-bound compartments. Each of these compartments is characterized by the presence of a specific set of resident proteins. It is now well established that the localization of integral membrane proteins to the various compartments of the endocytic and secretory pathways is mediated by information encoded within the structure of the proteins. In some cases, targeting information is contained within short, linear sequences of amino acid residues that act as specific sorting signals; the signals are recognized by receptor-like molecules. In other cases, the targeting information is a manifestation of a global physical-chemical property of the proteins, such as a tendency to partition into membranes with a particular lipid composition. Over the past year, our group has conducted studies on both types of mechanisms. Most of our work has focused on tyrosine-based sorting signals involved in internalization from the cell surface and lysosomal targeting and on adaptor complexes that function as recognition molecules for such signals. We have also studied the role of transmembrane domains in protein retention within the endoplasmic reticulum (ER), which may be mediated by lipid partitioning. Our recent progress on these projects is described in the following sections.

Molecular mechanisms involved in sorting integral membrane proteins to endosomes and lysosomes.

Many integral membrane proteins contain within their cytoplasmic tails tyrosine-based sorting signals that conform to the tetrapeptide motif YXXØ, where Y is tyrosine, X is any amino acid, and Ø is an amino acid with a bulky hydrophobic side chain. These signals mediate various sorting processes, including internalization from the cell surface and lysosomal targeting. We had previously demonstrated that the medium chains, μ_1 and μ_2 , of the clathrin-associated adaptor complexes, AP-1 and AP-2, function as the recognition molecules for tyrosine-based sorting signals. AP-2 is found in association with the plasma membrane and mediates internalization from the cell surface, whereas AP-1 localizes to the TGN where it mediates targeting of newly synthesized proteins to lysosomes. The involvement of tyrosine-based sorting signals in more than one sorting process raises the question of how tyrosine-based signals that conform to the same consensus motif can mediate sorting to different end compartments. In order to address this question we have examined the factors that determine the fine specificity and the avidity of signal-adaptor interactions. These studies have demonstrated that: (a) in general, μ_2 binds tyrosine-based signals with higher avidity than μ_1, (b) μ_1 and μ_2 display different preferences for residues surrounding the critical tyrosine residue, and © the position of the signal within the cytoplasmic tail of the proteins is critical for interactions; for instance, placement of the signal at the carboxy-terminus of the tail greatly increases the affinity of both μ_1 and μ_2 for the tyrosine-based signals. Thus, it is the combination of the sequence of the signal and its position within the tail that determines the overall strength of interaction with μ_1 and μ_2. Based on these findings, we have proposed a model according to which strong signals will bind to μ_1 in the TGN, resulting in lysosomal targeting. Weak signals, on the other hand, will not be recognized by μ_1, thus allowing for transport of the proteins to the cell surface. Once at the surface, the proteins will then be in a position to bind to the high affinity μ_2, leading to their internalization into endosomes.

Sequence requirements for interaction of tyrosine-based sorting signals with the adaptor medium chains determined by combinatorial methods.

The identification of the medium chains, μ_1 and μ_2, as the receptors for tyrosine-based sorting signals has enabled analyses of the properties of the signals at a molecular level. In order to establish the sequence requirements for interaction, we screened combinatorial libraries using μ_2 as a selector molecule. One of the approaches consisted of screening a combinatorial DNA library using the yeast two-hybrid system. In another approach, carried out in collaboration with the groups of Tomas Kirchhausen and Lewis Cantley (Harvard Medical School), combinatorial peptide libraries were screened *in vitro* with recombinant μ_2. Both approaches resulted in the identification of the sequence YXRL as the optimal signal for interaction with μ_2. Analyses of suboptimal sequences revealed that all of the selected peptides conform to the YXXØ motif defined previously from studies in vivo. We also examined the effect of mutations at the four positions on interactions with μ_2 by surface plasmon resonance spectroscopy. We found an excellent correlation between the apparent affinity of different signals for μ_2 *in vitro* and their sorting activity *in vivo*. These observations confirm the role of μ_2 as a receptor for tyrosine-based sorting signals and reveal additional properties of the interaction.

In vivo overexpression/saturation experiments suggest that tyrosine- and di-leucine-based sorting signals utilize different components of the sorting machinery

During the past year, we completed our studies on the saturability of signal-mediated protein sorting *in vivo*. The existence of specific recognition molecules that bind to tyrosine-based sorting signals predicts that processes mediated by such signals should be saturable. We tested this prediction by overexpressing proteins that have tyrosine-based signals and analyzing the surface expression of endogenous proteins that are normally targeted to lysosomes by virtue of tyrosine-based signals. We observed that overexpression interfered with several processes dependent on tyrosine-based signals such as lysosomal targeting and internalization from the cell surface. Interestingly, overexpression of proteins containing di-leucine-based signals, which are also involved in internalization and lysosomal targeting, did not compromise sorting mediated by tyrosine-based signals but did interfere with the sorting of other proteins containing di-leucine-based signals. These results suggest that tyrosine-based and di-leucine-based signals utilize distinct saturable components of the sorting machinery. Since tyrosine-based signals are known interact with μ_1 and μ_2, our findings most likely imply that di-leucine-based signals interact with either another subunit of the adaptor complexes or with a different type of protein. These studies were extended to the HIV-1 envelope glycoprotein complex, which also has several tyrosine-based signals that are active in endocytosis. Overexpression of the envelope glycoprotein, and of chimeric proteins derived from it, resulted in missorting of lysosomal proteins to the cell surface, a phenomenon that may explain some of the cytopathic effects of HIV-1 on the host cells.

A novel adaptor-like complex that recognizes tyrosine-based sorting signals.

In addition to internalization and lysosomal targeting, tyrosine-based signals have been implicated in various other sorting processes, including transport from early to late endosomes, delivery to specialized endosomal/lysosomal organelles such as MHC class II antigen-processing compartments, localization to the TGN, and targeting to the basolateral surface of polarized epithelial cells. The diversity of the processes mediated by tyrosine-based sorting signals makes it difficult to explain the function of the signals solely on the basis of interactions with the AP-2 and AP-1 adaptors. It is thus conceivable that there may be additional adaptor-like complexes that mediate some of the additional sorting functions attributed to tyrosine-based signals.

We have recently identified a novel adaptor-like complex that is a likely candidate for being another receptor for tyrosine-based sorting signals. The identification of this complex was made possible by the cloning of two novel cDNAs encoding proteins that have ~30% sequence identity to the adaptor small chains σ_1 and σ_2. The two proteins, which were named σ_{3A} and σ_{3B} , are very closely related to each other and are expressed in all cells and tissues examined. An antibody that recognizes both σ_3 proteins immunoprecipitates from fibroblasts a complex with a subunit composition similar to AP-1 and AP-2. The medium chain of this novel complex, known as p47A, also interacts with tyrosine-based signals. Experiments are now underway to establish the localization of this complex within cells; preliminary evidence suggests that it may associated, at least in part, with endosomes. We speculate that this novel complex might be involved in sorting proteins from early to late endosomes or from endosomes to the basolateral plasma membrane of polarized cells.

Role of transmembrane domains in protein sorting

All of the studies described above dealt with sorting events mediated by interactions between signals and their recognition molecules. Some sorting processes, however, do not rely on signals but depend on some physical-chemical property of the proteins. Several years ago, we demonstrated that transmembrane domains that either had charged or strongly hydrophilic amino acids or were too short caused retention of incompletely assembled proteins in the ER. In collaboration with Allan Weissman (NCI), we have now extended these observations to the ER resident protein UBC6. UBC6 is a cytosolically-oriented ubiquitin-conjugating enzyme attached to the ER membrane by a carboxy-terminal hydrophobic anchor. Molecular dissection of UBC6 revealed that the membrane anchor was both necessary and sufficient for ER localization. Moreover, we demonstrated that lengthening the hydrophobic span of the membrane anchor caused release from ER retention and allowed transport to the plasma membrane via the classical secretory pathway. Thus, we conclude that the carboxy-terminal membrane anchor of UBC6 is responsible for the retention of the protein within the ER and that the length of the transmembrane span is what determines its role in ER retention. We speculate that the mechanism by which the UBC6 membrane anchor mediates ER retention is related to that involved in protein localization to the Golgi complex. A current view of this mechanism is that short transmembrane domains may cause partitioning of proteins into the thinner membranes of the ER and the Golgi complex rather than into the thicker membranes of post-Golgi organelles.

Proposed Course of Research

The identification of the medium adaptor chains as the receptors for tyrosine-based signals has had a major impact in our ability to study the molecular mechanisms of protein sorting. We plan to continue the characterization of signal-adaptor interactions at a molecular level, making use of various binding assays developed in the course of our previous work. A major priority will be to establish the structural features of the signals that determine their role in various sorting processes, such as internalization, lysosomal targeting, TGN localization and basolateral targeting. We are currently conducting structure-function analyses of the medium chains, for the purpose of identifying the signal-binding domains, sites of interaction with other adaptor chains, regulatory regions, etc. This information on the medium chains will be used to construct dominant mutants that will allow us to study the function of these chains in intact cells. Another major focus of our research will be to establish the function of the newly discovered adaptor-like complex. If it is confirmed that this complex is associated with endosomes, we will examine whether it is involved in transport between early and late endosomes or in targeting to the basolateral surface of polarized epithelial cells. These studies will be extended to a model organism such as *Drosophila*, for which mutants of some of the adaptor subunits already exist. Finally, we will attempt the identification of proteins that bind to other sorting signals such as di-leucine motifs and acidic clusters using techniques similar to those that led to the identification of the medium adaptor chains as the receptors for tyrosine-based signals.

Publications.

Boll W, Ohno H, Songyang Z, Rapoport I, Cantley LC, Bonifacino JS, Kirchhausen T. Minimal sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes. EMBO J 1996;in press.

Bonifacino JS, Marks MS, Ohno H, Kirchhausen T. Mechanisms of signal-mediated protein sorting in the endocytic and secretory pathways. Proceedings of the Association of American Physicians 1996;108,in press.

Delahunty MD, Bonifacino JS. Disorders of intracellular protein trafficking in human disease. Conn Tissue Res 1995;31:283-286.

Delahunty MD, Rhee I, Freed EO, Bonifacino JS. Mutational analysis of the fusion peptide of human immunodeficiency virus type 1: identification of critical glycine residues. Virology 1996; 218:94-102.

Marks MS, Roche P, van Donselaar E, Woodruff L, Peters PJ, Bonifacino JS. A lysosomal targeting signal in the cytoplasmic tail of the beta chain directs HLA-DM to the MHC class II antigen processing compartment. J Cell Biol 1995;131:351-269.

Marks MS, Woodruff L, Ohno H, Bonifacino JS. Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. J Cell Biol 1996;in press.

Ohno H, Fournier MC, Poy G, Bonifacino JS. Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. J Biol Chem 1996;in press.

Ohno H, Kirchhausen T, Bonifacino JS. Recognition of tyrosine-based sorting signals by the medium chains of clathrin-associated protein complexes. J NIH Res 1995;7:50-51.

Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 1995;269:1872-1875.

Ooi CE, Rabinovich E, Dancis A, Bonifacino JS, Klausner RD. Copper-dependent degradation of the S. cerevisiae plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. EMBO J 1996;15:3515-3523.

Shapiro J, Sciaky N, Lee J, Bosshart H, Angeletti R, Bonifacino JS. Localization of endogenous furin to the Golgi complex. J Histochem Cytochem 1996;in press.

Voorhees P, Diegnan E, van Donselaar E, Humphrey J, Marks MS, Peters PJ, Bonifacino JS. An acidic sequence within the cytoplasmic domain of the furin functions as a determinant of TGN localization and internalization from the cell surface. EMBO J 1995;14:4961-4975.

Wienecke R, Maize Jr JC, Shoarinejad F, Vass WC, Reed J, Bonifacino JS, Resau JH, de Gunzburg J, Yeung RS, DeClue JE. Co-localization of the TSC2 product tuberin with its target Rap1 in the Golgi apparatus. Oncogene 1996;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-HD-01608-6

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene Regulation in Response to Environmental Stress

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Storz	Senior Staff Fellow	CBMB, NICHD
Others:	E. Bruggemann	IRTA	CBMB, NICHD
	K. Handwerger	Pre-IRTA	CBMB, NICHD
	C. Merlotti	Visiting Fellow	CBMB, NICHD
	A .Zhang	IRTA	CBMB, NICHD
	M. Zheng	IRTA	CBMB, NICHD

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Unit on Environmental Gene Regulation

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS: 5.75	PROFESSIONAL: 5.75	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This group studies how organisms sense environmental signals and transduce the signals into changes in gene expression and cell physiology. The group has continued their characterization of the two regulators central to the *Escherichia coli* response to oxidative stress: OxyR, a 34 kDa transcriptional activator which is switched on by oxidation, and OxyS, a novel 109 nucleotide RNA regulator which acts as an activator and repressor of gene expression. In addition to studying the mechanisms of OxyR and OxyS action, the group has begun to determine what known regulators are important to the response to hydrogen peroxide in *Saccharomyces cerevisiae*. Using *Arabidopsis thaliana* as a model system, the group is also characterizing mutants defective in their developmental response to blue light.

Z01 HD 01608-06

GENE REGULATION IN RESPONSE TO ENVIRONMENTAL STRESS

Project Description

Objectives:

This project is aimed at understanding the molecular mechanisms whereby organisms perceive an environmental signal and transduce this information into a change in gene expression. In one subproject, we are characterizing the *E. coli* and *S. cerevisiae* responses to oxidative stress, and in a second subproject, we are characterizing *Arabidopsis* mutants to elucidate how plant development is regulated by light.

Methods Employed:

Recombinant DNA techniques employed include the isolation of RNA and DNA, molecular cloning, nucleic acid electrophoresis, DNA sequencing, Northern and Southern blotting, chemical synthesis of deoxyoligonucleotides, DNA amplification via the polymerase chain reaction, and cDNA library construction and subtraction. Biochemical approaches used include protein purification, DNA binding and *in vitro* transcription assays, immunoblotting, and spectrometry. Standard genetic screens and crosses are also being carried out.

Oxidative Stress Responses in Bacteria and Yeast.

Reactive oxygen species ($O_2\bullet$, H_2O_2, and HO•) can lead to the damage of almost all cell components (DNA, lipid membranes, and proteins) and have been implicated as causative agents in several degenerative diseases. Most organisms have an adaptive response to defend against oxidants. Treatment of both bacterial and yeast cells with low doses of H_2O_2 results in the induction of distinct groups of proteins, the decreased expression of other proteins, and increased resistance to killing by subsequent higher doses of H_2O_2. In bacterial cells, a key regulator of the defenses against H_2O_2 is the OxyR protein. OxyR appears to be both the sensor and transducer of the oxidative stress signal, since the oxidized but not the reduced form of the purified regulator can activate transcription *in vitro*. H_2O_2 treatment also induces the expression of OxyS, a unique, non-translated RNA which has been shown to activate and repress as many as 40 genes in *E. coli*. The mechanism of OxyS action is now under investigation. Less is known about the induction of eukaryotic defenses against H_2O_2, but we have recently initiated studies to identify regulators of the *S. cerevisiae* response to oxidative stress.

Characterization of the redox-active center of OxyR

We previously isolated several non-binding and constitutively-active OxyR mutants. We are now studying the biochemical properties of wild type OxyR together with the mutant proteins to elucidate the nature of the redox-active center in OxyR. Metal analysis and experiments in which OxyR was denatured, dialyzed, and renatured, suggest that OxyR does not contain a metal co-factor. Therefore, we favor the hypothesis that the redox-active center of OxyS is Cys199 which is oxidized to a sulfonic acid upon exposure to H_2O_2. The status of this cysteine residue is now being probed with mass spectrometry and chemicals shown to react specifically with sulfonic acid. In addition, we have initiated studies to identify factors that re-reduce the OxyR protein in the cell.

Regulation by the OxyS RNA

We are continuing our studies of the novel, 109 nucleotide RNA which is induced by H_2O_2. Previously, we found that OxyS acts in *trans* to activate and repress the expression of a number of genes including *fhlA*, which encodes a transcriptional activator, and *rpoS*, which encodes a σ subunit of RNA polymerase. To gain insights into the mechanisms of OxyS action, we examined OxyS repression of *fhlA* and *rpoS*. OxyS acts at a post-transcriptional level for both of these genes and probably prevents *fhlA* translation by pairing with mRNA sequences adjacent to the ribosome binding site. The OxyS response site at *rpoS* has not been identified, but we have recently found that OxyS-mediated repression requires HF-I, a non-specific RNA binding protein. We are now testing whether OxyS binds HF-I or any other proteins. In parallel studies, we compared the levels of OxyS with the levels of other small stable RNA species and found that OxyS is abundant with as many as 4,000 molecules of OxyS per cell.

rpoS expression is regulated at multiple levels, and in the course of our studies of OxyS-mediated repression, we isolated a mutation in *rssB* which stabilized the RpoS protein. Interesting, RssB shows homology to response regulators and is the first such regulator shown to be involved in the control of protein turnover.

Regulators of the Yeast Response to Oxidative Stress

Relatively little is known about the eukaryotic defenses against oxidative damage. Therefore, we have also begun to examine the *S. cerevisiae* response to H_2O_2. To gain an overview of what known regulators are important for protection against H_2O_2, we constructed isogenic strains carrying mutations in *YAP1*, *YAP2*, *MAC1*, *POS9*, *MSN2*, and *MSN4*. We are comparing the viability of these mutant strains under different stress conditions and are examining the induction of antioxidant genes in the mutant strains. We have also begun to use the mutant backgrounds to isolate suppressor mutations in additional regulators and additional components of the oxidative stress signal transduction pathways.

Blue Light Regulation in *Arabidopsis*

The normal development of plants is dependent on light, and spectral studies have shown that plants undergo morphological changes in response red/far-red, blue, and UV light. For example, dark grown plants have extremely elongated hypocotyls (stems) compared to seedlings grown under red or blue light. At present, the mechanisms by which the light signals are transduced into changes in plant growth and morphology are largely unknown.

Characterization of *hy4* and *hy5* mutants

We took advantage of the light-effect on hypocotyl length to screen for *Arabidopsis* mutants that have elongated hypocotyls under blue light. 24 independent mutants defective in their developmental response to blue light were isolated from a screen of 300,000 fast neutron-mutagenized seeds. The mutants fell into two complementation groups corresponding to the *HY4* and *HY5* genes. Since *HY4* is thought to encode the blue light photoreceptor, we characterized the 21 *hy4* mutants to determine the nature of the mutations. We found 20 of these fast neutron-generated mutants displayed straightforward genetic behavior and had detectable mutations at the *HY4* locus. Many of the mutations were large deletions demonstrating that fast neutron irradiation is an effective means of generating deletions suitable for genomic subtraction. The last *hy4* mutant showed extremely interesting genetic behavior; the strain exhibited a wild type phenotype in one generation and a mutant phenotype in the next. Since we have not been able to detect a molecular defect at the *HY4* locus, we propose that this line has an epigenetic mutation, the first such mutation to be identified in *Arabidopsis*. These studies of the *hy4* mutants are being complemented by subtractive screens between wild type, *hy4*, and *hy5* cDNA libraries to identify genes that are differentially expressed between the wild type and mutant seedlings.

Publications

Bruggemann E, Handwerger K, Essex C, Storz G. Analysis of fast neutron-generated mutants at the *Arabidopsis thaliana HY4* locus. Plant J 1996;in press.

Muffler A, Fischer D, Altuvia S, Storz G, Hengge-Aronis R. The response regulator RssB controls stability of the σ^s subunit of RNA-polymerase in *Escherichia coli*. EMBO J 1996;15:1333-1339.

Shirley BW, Kubas WL, Storz G, Bruggeman E, Koornneef M, Ausubel FM, Goodman HM. Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. Plant J 1995;8:659-671.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-HD-01609-05

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Localization and Dynamics of Intracellular Organelles

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Lippincott-Schwartz	Senior Staff Fellow	CBMB, NICHD
Others:	N. Cole	Pre-IRTA	CBMB, NICHD
	J. Ellenberg	Special Volunteer	CBMB, NICHD
	J. Presley	IRTA	CBMB, NICHD
	K. Zaal	IRTA	CBMB, NICHD

COOPERATING UNITS *(if any)*

University of Connecticut (Mark Terasaki); Johns Hopkins University (Michael Edidin); Cornell University (Eric Siggia); NIMH, LCM, NIH (Carolyn Smith).

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Unit on Organelle Biology

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.8	2.8	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

How membrane-bound organelles of eukaryotic cells maintain their identity and subcellular localization amidst an enormous inpu and outflow of membrane and protein is a central question in cell biology. Studies in this group have focused on this question and have sought to define the cellular and molecular mechanisms which underlie the organization and distribution of eukaryotic organelles. Particular attention has been paid to the Golgi complex which plays a fundamental role in the processing and sorting of protein moving through the secretory pathway. The Golgi complex in higher eukaryotes consists of stacks of flattened cisternae usually localized to the perinuclear region near the microtubular organizing center (MTOC). Recent studies have suggested this organization and positioning of the Golgi are controlled by dynamic processes. Tubulovesicular structures emerging from Golgi elements along microtubules, for example, enable adjacent Golgi stacks to communicate. In addition, reversible dispersal of Golgi elements occurs during microtubule disruption, mitosis and brefeldin A (BFA)-treatment. To further understand these processes and their relationship to the three-dimensional morphology and function of the Golgi we have generated Golgi protein chimeras containing the green fluorescent protein (GFP). Using these constructs to label Golgi complex in living cells, we have performed time-lapse imaging to examine the dynamics of the Golgi complex in under a variety of conditions. In addition, we have examined the diffusional mobility of the chimeras in interphase and mitotic cells. Results from these studies are providing new insights into mechanisms of Golgi protein retention and targeting.

LOCALIZATION AND DYNAMICS OF INTRACELLULAR ORGANELLES

Project Description

Objectives:

The unit on Organelle Biology focuses on issues related to the control of organelle structure and dynamics within cells. Over the past year this group has focused on understanding the dynamics of membrane proteins which comprise the ER/Golgi membrane system, which plays a central role in the biosynthesis, transport, processing and sorting of secretory products within the cell. Toward this goal, protein chimeras containing the green fluorescent protein (GFP) have been generated which localize within the ER/Golgi membrane system and have been used to examine the dynamics and diffusion rates of these proteins in interphase and mitotic cells.

Probing the diffusional mobility of Golgi protein-GFP chimeras:

The Golgi complex contains a large number of resident components that play important roles in membrane processing and sorting. How these proteins are retained in the Golgi despite a continuous flow of protein and lipid through the secretory pathway is not yet understood. To address this question the diffusional mobilities of mammalian Golgi membrane proteins (including galactosyltransferase, mannosidase II and KDEL receptor) fused to GFP were measured in living HeLa cells. This work utilized fluorescence photobleaching recovery (FPR) and fluorescence loss in photobleaching (FLIP) techniques to measure the diffusion rates of GFP-chimeras in Golgi membranes and to examine the extent of lateral membrane continuity between Golgi stacks. The results from this work revealed rapid and extensive diffusion of all GFP-Golgi chimeras within Golgi membranes, indicating Golgi retention of these proteins does not depend on protein immobilization. Rapid and widespread loss of fluorescence from the Golgi upon repeated bleaching of a small zone within the complex, furthermore, implied significant lateral diffusion rather than vesicular transport of the GFP-tagged proteins between Golgi stacks. These results raise the question of how Golgi membranes maintain their identity amidst rapid diffusion of resident components and what role such dynamic properties play in Golgi membrane structure and function.

Diffusional mobility of Golgi proteins in mitotic cells

The Golgi apparatus undergoes a profound reorganization during mitosis in many cell types and is widely assumed to vesiculate into thousands of small vesicles scattered throughout the cytoplasm, which remain inactive until the end of mitosis when they actively fuse to regenerate Golgi stacks. Ultrastructural studies of mitotic cells have supported this thinking by showing Golgi proteins in vesicle clusters. However, Golgi proteins have also been observed in the continuous membranes of the ER during mitosis, raising the possibility that these proteins are not restricted in their localization to static vesicles. To investigate this possibility the diffusional mobility of Golgi proteins tagged with GFP were examined in mitotic HeLa cells. Using confocal microscopy a narrow box across the edge of a mitotic cell expressing galactosyltransferase (Galtase)-GFP was repeatedly bleached with high power laser light and areas outside the box were monitored for fluorescence loss in photobleaching (FLIP). Galtase-GFP fluorescence associated with the entire cell was lost with a half-time of 2 min indicating that all of the chimeras were highly mobile and could diffuse rapidly into the narrow photobleached zone. The rate of loss of Galtase-GFP fluorescence was significantly faster than that of rhodamine transferrin or rhodamine dextran observed by FLIP, and resembled that of ER-localized GFP chimeras. These results suggest that Golgi proteins in mitotic cells are not restricted in their localization to static vesicles, but are diffusing rapidly within an interconnected membrane system, which most likely includes the ER.

Kinetics of brefeldin A-induced resorption of Golgi into ER: evidence of flow-assisted membrane transport

GFP-Golgi chimeric proteins were used to examine the dynamics of the Golgi complex in vivo and how it is perturbed by brefeldin A (BFA). BFA dramatically alters the distribution and transport of membrane through organelles by blocking an essential activation step in the membrane interaction of a family of closely related proteins called ADP ribosylation factors, which are low molecular weight GTP-binding proteins. In control cells imaged at 3 sec time intervals with a confocal microscope system, thin membrane tubules were found to rapidly extend out and disconnect from stable perinuclear Golgi elements. Detached, mobile tubule elements containing the GFP chimeras moved away from the Golgi along curvilinear microtubule tracks at rates of 0.6 um/sec before changing shape and direction. Upon BFA-treatment, tubule processes appeared more frequently and failed to detach from Golgi structures. A dynamic tubule network extending through the cytoplasm was soon generated and persisted for 5-10 min until rapidly emptying its contents into the ER within 15-30 sec. Both lipid and protein emptied from the Golgi at similar rapid rates leaving no Golgi structure behind. The kinetics of the redistribution process was followed and compared with numerical simulations of diffusion in two dimensions that take account of the actual Golgi shape and the observed density of ER membrane. A large discrepancy between simulation and experimental data was found suggesting an alternative model for Golgi redistribution into the ER that is analogous to wetting or absorption phenomenon where a membrane flow supplements diffusive transfer. Such flow-assisted redistribution of Golgi into ER during BFA treatment suggests that mechanisms which regulate retrograde tubule formation and detachment from the Golgi are integral to the existence and maintenance of this organelle.

ER-to-Golgi transport of VSVG-GFP chimeras in living cells

The nature of the transport intermediates that carry proteins between the ER and Golgi complex is currently unclear. Two major models have been proposed. In one, proteins move through a long-lived 'intermediate compartment' which continually packages new transport vesicles for transport into the Golgi complex. In the second, cargo-containing vesicles bud from the ER and fuse to form larger pleiomorphic structures (i.e. intermediate compartment) which themselves translocate to and fuse with the Golgi. To distinguish between these models, the ts045 mutant of VSVG protein was tagged with GFP (VSVG-GFP) and expressed in Cos cells. VSVG-GFP has the property of misfolding and being retained in the ER at 40°C. This property is rapidly reversible upon shift to 32°C, permitting the release of a synchronized pool of protein to the Golgi complex. ER-to-Golgi transport of VSVG-GFP was followed using time-lapse imaging of cells on a fluorescent microscope equipped with a cooled CCD camera and filters optimized for GFP. VSVG-GFP accumulated in tubulovesicular structures (TVS) scattered throughout the cell with shift from 40°C to 16°C for two hours. These TVS resembled structures previously described as the intermediate compartment and contained ßCOP. Upon shift from 16°C to 32°C, TVS translocated rapidly as units into the centrosomal region and fused with the Golgi complex. TVS moved along straight or curvilinear paths at speeds up to one micron per second, consistent with a microtubule minus end motor powering their movement. A single long tubule was often seen extending in the direction of motion from a motile TVS, suggesting that a motor on the tip of the tubules was pulling the TVS towards the Golgi. This motility did not occur after microtubule disruption with nocodazole. Upon shift directly from 40°C to 32°C similar fast movement of TVS from peripheral sites into the centrosomal region was observed. These results favor a model for ER-to-Golgi transport in which the intermediate compartment itself translocates to and fuses with the Golgi complex.

Golgi-to-ER transport of VSVG chimeras

Proper functioning of the secretory pathway in higher eukaryotes requires a significant retrograde flow of membrane and protein from the Golgi complex back to the endoplasmic reticulum (ER). This ensures return of escaped ER resident proteins, as well as membrane components necessary for continued anterograde traffic. A variety of molecules have been shown to cycle constitutively between the ER and Golgi complex, although the exact routes these molecules travel is unclear. Whether Golgi resident proteins cycle through these pathways, as well, is unknown. To address this question we constructed chimeric Golgi proteins containing the luminal domain of the temperature sensitive viral glycoprotein, tsO45VSV G. We reasoned that if Golgi-targeted VSV G chimeras were to cycle between the ER and Golgi complex, then shifting the temperature to 40°C should result in their misfolding and retention in the ER as they cycled through this compartment. In transiently transfected cells at 32°C, VSVG chimeras (including VSV G attached to the transmembrane and cytoplasmic domains of TGN 38, KDELR and KDELR_m) were found localized to the Golgi complex. Upon shifting the temperature to 40°C, these proteins redistributed into the ER, without altering the distribution of other Golgi membrane markers. The kinetics of this process varied with each chimera, with complete redistribution occurring in as little as fifteen minutes, or as long as three hours. This process was fully reversible, as all chimeras showed a Golgi staining pattern within one hour of shift back to the permissive temperature. These observations suggest that there is extensive retrograde traffic of Golgi membrane proteins from the Golgi back the ER.

Publications

Lippincott-Schwartz J. Membrane traffic and compartmentalization within the secretory pathway. In: Schultz et al, eds. Molecular biology of membrane transport disorders. Plenum Press, 1996;1-9.

Cole NB, Sciaky N, Marotta A, Song J, Lippincott-Schwartz J. Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral ER exit sites. Mol Biol Cell 1996;7:631-650.

Cole NB, Smith C, Sciaky N, Terasaki M, Edidin M, Lippincott-Schwartz J. Diffusional mobility of Golgi proteins in membranes of living cells. Science 1996;273:797-801.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-HD-01610-04

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Intracellular Metal Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Richard D. Klausner, M.D., Chief, CBMB

Others:	A. Dancis	Expert	CBMB
	A. Finegold	NRC	CBMB
	Y. Iwai	Visiting Fellow	CBMB
	C. Philpott	Medical Staff Fellow	CBMB
	R. Stearman	Biologist	CBMB
	D. Yuan	Medical Staff Fellow	CBMB

COOPERATING UNITS *(if any)*

Whitehead Institute for Biomedical Research, Cambridge, MA (G. Fink); Dept. of Biochemistry, SUNY Buffalo, Buffalo, NY; Dept. of Medicine, University College, London, UK (A. Segal)

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Section on Organelle Receptor Structure and Function

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.85	4.85	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This group has continued to work on metal homeostasis in *Saccharomyces cerevisiae*. The FRE1 gene of yeast is similar to the gp91-*phox* subunit of the NADPH oxidase of human granulocytes. We have studied structure-function relationships of the FRE1 gene, in particular identifying the heme-liganding residues which are probably conserved with the human homolog. We have obtained additional evidence for an iron transport complex present in the yeast plasma membrane and consisting of the multi-copper oxidase, FET3 and the permease, FTR1. CCC2, P-type ATPase similar to the Wilson disease and Menkes disease genes of humans, is required for copper delivery to FET3. The copper-loading function of CCC2 was localized to a post-Golgi compartment by making use of a panel of sec (secretory) and vps (vacuolar protein sorting) mutants with defects in iron uptake and copper loading of FET3. AFT1, the activator of iron transport was shown to interact with a specific DNA sequence, thereby transducing the signal for iron deprivation. Analogously, MAC1 was shown to interact with a distinct target sequence, transducing the signal for copper deprivation. The cellular effects of iron overload were evaluated and found to include a cell cycle arrest in both G_1 and G_2. Evaluation of excess iron uptake in strains deficient in DNA repair (*rad1, rad6, rad52,* and *apn1*) suggests that the G_2 arrest is due to DNA damage, while the$_1$G arrest is due to downregulation of G cyclins. Homologs of the FTR1 iron permease have been identified in other organisms, including *B. subtilis*, *S. pombe*, and *C. albicans*. The *C. albicans* homolog has been cloned by complementation of the FTR1 mutant. A human homolog of the FTR1 iron permease is being sought by complementation. Finally, zinc regulated genes (both induced and repressed) have been identified by screening random lacZ fusions inserted in the yeast genome.

INTRACELLULAR METAL METABOLISM

Project Description

Objectives

(1) To use the methodology of yeast genetics to identify genes involved in the regulated uptake and utilization of iron, copper and zinc, (2) to define structural and regulatory relationships among these genes, (3) to determine how cellular metals are "sensed" and how metal-dependent gene regulation ensues from this, and (4) to investigate the toxic consequences of disturbance of the iron sensing pathway. Particular attention has been given to insights that may be gained through study of the yeast homologs of human disease genes.

Major Findings

Identification of the heme-coordinating residues conserved between FRE1 and gp91-*phox*. Identification of a novel family of membrane cytochromes

The initial step in cellular iron acquisition by *Saccharomyces cerevisiae* (and most likely in humans) involves the reduction of iron chelates in the environment by an externally directed surface reductase. The major iron reductase of yeast is encoded by the *FRE1* gene. FRE1 bears sequence similarity to gp91-*phox*, the large subunit of the NADPH oxidase of human granulocytes, and the defective component in most patients with the childhood immune deficiency, chronic granulomatous disease. In collaboration with the laboratory of Dr. Anthony Segal, we have shown that FRE1, like gp91-*phox*, is a b-cytochrome containing hemes with low mid-point potential (about -250mv). Four critical histidine residues in the FRE1 protein of the yeast reductase (H294, H308, H364, H378) were identified by site directed mutagenesis. The members of each pair were separated from each other by 13 predominantly hydrophobic amino acids. Individual mutation of each histidine to alanine eliminated reductase activity and the entire heme spectrum. We considered that the failure to insert heme into FRE1 might destabilize the protein making it difficult to distinguish mutations affecting heme liganding from mutations affecting protein stability. However, levels of the apoprotein in the mutant strains lacking a heme spectrum were comparable to levels of the holoprotein in the wild-type strain. Therefore effects of these mutations in abrogating the heme spectrum are likely to be specific and not mediated by general effects on protein stability.

These critical residues are predicted to coordinate a bis-heme structure between transmembrane domains of the FRE1 protein. The histidine residues are conserved in the related gp91-*phox* protein of the NADPH oxidase of human granulocytes, predicting the sites of heme coordination in that protein complex which have not been previously identified. Similarly spaced histidine residues have also been implicated in heme binding by organelle cytochromes with little overall sequence similarity to the plasma membrane b cytochromes. The shared bis-heme binding motif raises the question of the evolutionary and functional relationships between the plasma membrane proteins gp91-*phox* and FRE1, and the organelle b cytochromes. At the level of the primary amino acid sequence only the spacing of the histidines in a generally hydrophobic context is conserved between these two groups of proteins. However, a more closely related protein family including FRE1 and gp91-*phox* can be defined. The members of this family possess hydropathy profiles with several (six to eight) hydrophobic domains at the amino terminus. The bis-heme binding motif occurs in this region. A hydrophilic carboxy terminal domain present in these proteins and absent in the organelle b cytochromes contains a number of conserved amino acid motifs occurring in the same order and with similar spacing, that may relate to cofactor binding (NADPH and FAD). This protein family includes the homologs of the gp91-phox gene from various other species including pigs, mice, and even plants, and these genes presumably function as part of a superoxide generating complex in cellular defense. A BLAST computer search of the recently completed *Saccharomyces cerevisiae* genome database for homologs of FRE1 identified FRE2 and seven additional open reading frames that appear to belong to the same gene family. These newly identified yeast genes resemble FRE1 to varying degrees but show little similarity to the organelle b cytochromes. The role of the bis-heme binding motif will be further clarified by definition of the cellular localization and function of the members of this gene family. It is highly likely that transmembrane electron transport will be involved in the function of these genes.

Permease-oxidase complex involved in high-affinity iron transport

The reduced iron generated by the activity of the externally directed reductase is assimilated into the yeast cell by the activity of a high-affinity transport system. We have reported previously on the identification of the a gene encoding the iron permease. This gene was initially called FTS3 for FerrousTransport Suppressor, because it was identified as an extragenic suppressor of the high iron uptake phenotype of the mutant MA15 locus. However, the gene name has been changed to FTR1, for Ferrous TRansporter. FET3 encodes a multi-copper oxidase which is also required for high-affinity cellular iron uptake. The phenotypes of FTR1 and FET3 mutants were noted to be similar (see previous Annual Report), and evidence for interaction between these two gene products was obtained. In order to increase iron uptake above the baseline observed in the wild-type strain, high copy-number plasmids containing both FET3 and FTR1 had to be simultaneously present. Secondly, deletion of FTR1 abrogated FET3 oxidase activity. Because the FET3 oxidase activity could be restored in vitro by addition of copper to the lysate, the defect appeared to be a consequence of failure to insert copper into the FET3 protein. This copper-reconstituted FET3 oxidase activity migrated aberrantly on a gel. However, after treatment of the extracts with endoglycosidase H, the mobility of the copper-reconstituted oxidase was indistinguishable from that of the control. An explanation for these findings could be that in the ftr1 mutant the FET3 protein is mislocalized resulting in aberrant glycosylation. In contrast, failure to activate FET3 oxidase due to copper deficiency did not result in abnormal FET3 glycosylation suggesting that FTR1 is required both for maturation of FET3 through the secretory pathway and its acquisition of copper.

Thus, effects of FTR1 expression on FET3 cellular localization were observed, and conversely, effects of FET3 expression on FTR1 localization were observed. In the presence of an intact *FET3* gene, the epitope-tagged FTR1 was visualized as a bright rim of immunofluorescence consistent with a plasma membrane localization. However, in the absence of an intact *FET3* gene, the epitope-tagged FTR1 could be seen as an internal perinuclear ring, consistent with retention of the protein in the endoplasmic reticulum. Copper deprivation or mutation of the CCC2 gene, involved in copper delivery to FET3, did not give this pattern, but rather allowed surface localization of FTR1 protein to be achieved. In sum, then the genetics and the reciprocal effects of FET3 and FTR1 on localization suggest the existence of a protein complex at the plasma membrane. This would be consistent with the demonstrated role of the two components in high-affinity iron uptake. However, direct evidence of physical association of the components of the complex has still not been obtained.

Localization of CCC2 gene product by evaluation of a panel of sec and vps mutants

The cellular processes affected in two human genetic disorders of copper metabolism, Menkes disease and Wilson disease, involve the export of copper into the secretory pathway by putative copper-transporting P-type ATPases, but this export activity has been difficult to characterize. The CCC2 gene product of yeast is homologous to the human ATPases and also appears to function in copper export, since CCC2 protein is required for the delivery of cytosolic copper to the FET3 gene product, a copper enzyme required for high-affinity iron uptake whose copper binding domains lie outside of the cytosol. Sec mutants with temperature sensitive blocks in the secretory pathway were labeled with radioactive copper to show that Fet3p apopropein must exit the Golgi apparatus before it is loaded with copper. sec7, sec18, and sec23 mutants failed to insert copper into Fet3p when labeled at the non-permissive temperature, whereas sec 4 and sec1 mutants labeled normally. These results localize CCC2-dependent intracellular copper export to a post-Golgi compartment Several mutants with post-Golgi defects in vacuolar protein sorting were found to have iron uptake defects similar to those in ccc2 mutants, a finding that explains the long-recognized role of vacuolar biogenesis in mitochondrial respiratory function.

Cellular iron homeostasis - role of AFT1

Iron deprivation of *Saccharomyces cerevisiae* induces transcription of genes required for high-affinity iron uptake. AFT1 mediates this transcriptional control. The 5' flanking region of *FET*3 was analyzed and found to contain a DNA sequence responsible for AFT1-regulated gene expression. AFT1 was capable of interacting specifically with this DNA sequence. *In vivo* footprinting demonstrated occupancy of the AFT1 binding site in cells deprived of iron and not in cells grown in the presence of iron. Thus, the environmental signal resulting from iron deprivation was transduced through the regulated binding of AFT1 to the *FET3* promoter, followed by activation of transcription.

A regulon of genes under the control of AFT1 could be defined. AFT1 was able to bind to a consensus binding site (PyPuCACCCPu) in the 5' region of genes involved in iron acquisition.

These genes include FRE1 and FRE2, responsible for the surface iron reductase, and FET3 and FTR1, responsible for transmembrane transport of the reduced iron substrate. Interestingly, CCC2 was also found to be regulated by AFT1 and to possess an AFT1 binding site in its promoter region. CCC2 encodes a P-type ATPase required for copper delivery to FET3, which in turn is required for iron uptake. The AFT1 regulation of the CCC2 transcript is consistent with the role of this gene in iron metabolism.

Finally, a homolog of FTR1 was identified by data base searching. This gene, called FTH1 (ORF YBR207w), was found to possess an AFT1 regulatory site which was shown to interact with AFT1 protein by mobility shift assay. Moreover, the FTH1 transcript level was found to be regulated by iron and by AFT1. Iron deprivation induced FTH1 expression and iron loading repressed expression. Also, FTH1 expression was virtually undetectable in the Δaft1 strain. However, in the presence of the dominant AFT1-1up allele, iron regulation was still observed. This is in contrast with the other iron-regulated genes examined, and suggests that an additional AFT1-independent iron sensing/regulation pathway may exist. The FTH1 gene has been deleted and overexpressed, and a phenotype has not been discerned. Therefore, definition of a function for this gene must await further work.

Cellular copper homeostasis - role of MAC1

The FRE1 promoter fused to a HIS3 reporter was used to select mutants which fail to repress the promoter activity. Among these mutants were dominant alleles of AFT1 and another dominant mutant, called MA20. The following phenotypes were noted for the MA20 mutant: (1) high surface reductase poorly repressed by iron (2) elevated copper uptake and (3) copper sensitivity. These phenotypes were similar to phenotypes of the previously described MAC1up1 mutant. By performing the appropriate crosses and analyzing the tetrads, the mutation in MA20 was shown to be allelic with MAC1.

MAC1 was known to be a nuclear protein, and because of the copper-related phenotypes, we considered that it might be the regulator controlling expression of CTR1, the plasma membrane copper transport protein. Consistent with this hypothesis, deletion mutants of MAC1 and deletion mutants of CTR1 exhibited similar phenotypes - lack of high-affinity iron uptake which could be corrected by addition of copper (50μM copper sulfate to YPD) to the growth medium. A candidate target sequence for the MAC1 regulator was identified by deletion analysis of the CTR1 promoter, and interestingly this sequence included a palindrome containing the nucleotides TTTGCTCA. This sequence was noted to be identical to a sequence appearing as a direct repeat in the FRE1 promoter. Finally, a gel shift assay was established using MAC1 protein synthesized in vitro. In this assay, a CTR1 oligonucleotide derived from the regulatory site was specifically shifted by the MAC1 protein. The signal could be competed by the FRE1 direct repeat sequence. Thus, MAC1 appears to be a DNA interacting protein which most likely functions to transduce a cellular copper signal to FRE1 and CTR1, genes involved in copper homeostasis. Although FRE1 appears to be regulated by both AFT1 and MAC1, CTR1 is regulated by MAC1 alone and not by AFT1, consistent with its role in copper metabolism.

Iron intoxication: Implications for regulation of the cell cycle

The human disease hemochromatosis is a genetic disorder caused by excess uptake of iron from the diet, resulting in multi-organ failure and, often, liver cancer. Although a candidate gene for this disorder was recently identified, the molecular events through which iron causes damage to cells are largely unknown. We have investigated the toxic effects of excess iron accumulation in *S. cerevisiae* by using a dominant mutant allele of an iron regulator and found evidence of iron-mediated regulation of the cell cycle. Overexpression of a dominant mutant allele of *AFT1*, *AFT1-1*^{up}, results in high levels of ferrous iron uptake that is associated with a growth arrest and decreased survival in the presence of excess iron. Deletion of a component of the high affinity iron transport complex, *FET3*, relieves the growth impairment at high iron concentrations. *AFT1-1*^{up} strains grown in excess iron arrested with a high proportion (85%) of large, unbudded cells and flow cytometry reveals an accumulation of cells with 1N DNA content, consistent with a cell cycle arrest in G$_1$ or early S phase. Treatment of iron-arrested cells with mating pheromone demonstrated that the small fraction of cells with 2N DNA content is resistant to alpha factor, suggesting that a cell cycle arrest in both G$_1$ and G$_2$ occurs. The G$_2$ block is exacerbated by deletion of the DNA repair genes *RAD1*, *RAD6*, *RAD52*, and *APN1*, and is dependent on the DNA damage checkpoint gene, *RAD9*. Expression of *AFT1-1*^{up} in strains deficient in DNA repair causes dramatic reductions in survival and markedly slowed growth. Expression of *AFT1-1*^{up} is mildly recombinogenic and mutagenic. These data provide evidence indicating that iron intoxication leads to DNA damage, which activates a cell cycle checkpoint and results in a cell cycle arrest in G$_2$.

The G$_1$ arrest associated with iron intoxication is due to down- regulation of the G cyclins CLN1 and CLN2 by iron. This results in decreased activity of the CDC28 kinase and failure to progress through Start. Whether the down-regulation of G$_1$ cyclins occurs through alteration of protein half-life or through transcriptional regulation is an area of current research. The yeast gene GRR1 is a regulator of glucose transport and is required for efficient degradation of the G$_1$ cyclins. A *grr1* strain progresses rapidly through G$_1$ and microscopically appears with multiple elongated buds. Expression of *AFT1-1*^{up} in this strain results in a reversal of both the cell cycle and morphologic phenotypes, which is consistent with the iron-mediated down-regulation of CLN1 and CLN2. Strains expressing a hyperstable form of the G$_1$ cyclin CLN3 (*cln3-2*) also progress rapidly through G$_1$, but expression of *AFT1-1*^{up} in this strain fails to produce a cell cycle arrest. The effect of iron on CLN1 and CLN2 levels is being investigated in the *CLN3-2* strain. The G$_1$ arrest appears to be at least partially independent of DNA damage (it is not dependent on RAD9 and is not affected by mutations in DNA repair pathways). This raises the intriguing possibility that the eukaryotic cell cycle in regulated by iron or oxidative stress in a specific fashion. Mutant strains that demonstrate reduced survival in the presence AFT1-1up have been isolated and experiments are underway to isolate a subset of mutants that demonstrate alterations of the cell cycle phenotypes. This area of research will provide new insights as to how environmental signals are integrated into the regulation of the cell cycle.

Studies of zinc metabolism using yeast genetics

Like iron and copper, zinc is a trace element indispensable to most organisms. Although the importance of zinc to biochemistry is well known, little is known about how cells take up and distribute this essential nutrient. Such mechanisms are likely to be homeostatically regulated, since zinc is also toxic in excess. In addition, the secondary physiological consequences of zinc deficiency and zinc toxicity are poorly understood at the biochemical level. This knowledge is especially needed for a better understanding of human nutritional zinc deficiency and its many consequences, including birth defects and immunodeficiency.

Over the past year, many of the same genetic approaches that have proved useful for the analysis of iron and copper metabolism have been extended to studies of zinc. A genetic screen for zinc-regulated genes in yeast has been carried out, based on the assumption that important genes in zinc metabolism are zinc-regulated. This assumption was in turn grounded in the knowledge that mechanisms of iron and copper uptake are transcriptionally regulated. Random lacZ fusions were introduced into the yeast genome by transposon-mediated mutagenesis, using a library of such fusions created in the laboratory of M. Snyder (Yale University). Translational fusions involving zinc-regulated genes were then identified by isolating clones exhibiting zinc-regulated lacZ expression. Several technical modifications were introduced that greatly expedited this procedure. To date, 36 zinc-regulated genes have been identified, of which 12 exhibit over 20-fold regulation by changes in extracellular zinc availability.

Current efforts are focused on gaining a clearer understanding of the function of a putative transport protein identified in the genetic screen. This protein contains six transmembrane domains and several potential metal-binding sites, and importantly, is highly induced (>100-fold) in response to zinc deprivation. Loss of function mutations appear to result in a phenotype of flocculent growth that is also characteristic of growth of wild-type strains in low-zinc medium, suggesting that this transporter functions in zinc uptake or zinc utilization. Among the other zinc-regulated genes identified in this screen are those encoding the zinc-resistance protein Zrc1p, two zinc-finger proteins, the sodium transporter Pmr2p, and the widely distributed yeast retrotransposon, Ty1. Tools for the systematic study of these and other genes are now being tested and are anticipated to lead rapidly to many new insights into how eukaryotic cells respond to zinc deprivation and toxicity.

Relevance to human physiology and disease

The genetic analysis of the iron uptake system in yeast has continued to yield insights that may be relevant to human physiology and disease. Some of the yeast genes implicated in iron and copper metabolism are homologous to human genes. FRE1, a structural gene required for the predominant yeast iron reductase, is homologous to gp91-*phox*, a structural gene of the NADPH oxidase of human granulocytes. The residues within the human oxidase where the essential heme cofactors are bound have not been defined despite efforts by several laboratories. These residues could be identified in the yeast predicted protein, and extrapolation of this information predicts the sites of heme coordination in the human protein (see above).

The yeast CCC2 gene is homologous to the human Wilson and Menkes disease genes. Localization of the yeast gene product (see above) may have implications for the mechanism of action and the cellular site of action of the Menkes and Wilson disease gene products.

The CCC2 protein functions in the delivery of the copper to FET3, a multi-copper oxidase required for iron uptake. Analogously, the Wilson disease gene functions in the delivery of copper to ceruloplasmin. Ceruloplasmin has been implicated in iron metabolism in humans, and inactivating mutations in this gene lead to iron accumulation in the basal ganglia. Many questions remain, however. An iron import complex such as the FET3/FTR1 complex proposed for *Saccharomyces cerevisiae* has not been described in humans. Moreover the problems resulting form copper deficiency or inactive ceruloplasmin relate primarily to export of iron from the cells. It is possible that ceruloplasmin could interact with distinct membrane components to mediate export and/or import of iron. Alternatively, a novel multi-copper oxidase might function in iron uptake as part of a complex with a still unidentified permease.

We have begun to look for the human homolog of the FTR1 gene. Homologs in *B. subitilis* and *S. pombe* have been identified by DNA sequencing, although there is no biochemical data on the corresponding genes. A homologous gene in *S. cerevisiae* (*FTH1*) was identified as part of the genome project, and we have studied the iron-regulated expression of this gene (see above), although the deletion strain has no apparent phenotype. As part of a collaboration with the laboratory of Dr. Gerald Fink, we have used a library of genomic DNA from *Candida albicans* to complement the *FTR1* deletion strain of *S. cerevisiae*. The sequence of the complementing clone contains an ORF with more than 50% amino acid identity with the FTR1 protein. Comparison of the sequences of these FTR1 homologs and data from site directed mutagenesis of a motif (REGLE) suggested that this conserved sequence is critical for the iron transport function of FTR1.

Approaches to identifying the human homolog of FTR1 include 1) PCR based on degenerate sequences from conserved regions of FTR1 predicted protein and 2) functional complementation of the growth defect of the FTR1 mutant on iron depleted (ferrozine) plates. For this purpose, a cDNA library is being generated for insertion into a yeast expression vector under the control of the powerful PGK promoter.

The putative zinc transport protein identified in yeast contains structural motifs that appear to be conserved in several human homologs, including distinct variants isolated from liver and placenta. As nothing is presently known about the molecular basis of zinc transport in humans, these homologs will obviously receive careful scrutiny in the near future. A major potential application of this work is the development of sensitive diagnostic markers for zinc deficiency, analogous to the use of transferrin receptor assays for determination of iron status. Such definitive markers are currently lacking in the field and can be expected to have substantial clinical utility, given the wide prevalence of zinc deficiency in diarrheal diseases, malnutrition, and pregnancy. Studies of the pathophysiology of zinc deficiency and zinc toxicity in yeast may also provide heuristic guidance for analogous studies in human tissues.

Publications:

Shatwell KP, Dancis A, Cross AR, Klausner RD, Segal AW. The FRE1 ferric reductase of *Saccharomyces cerevisiae* is a cytochrome *b* similar to that of NADPH oxidase. JBC 1996;271:14240-14244.

Stearman R, Yuan DS, Yamaguchi-Iwai Y, Klausner RD, Dancis A. A permease-oxidative complex required for high-affinity iron uptake in yeast. Science 1996;271:1552-1557.

Yamaguchi-Iwai Y, Stearman R, Dancis A, Klausner RD. Iron-regulated DNA binding by the AFT1 protein controls the iron regulation in yeast. EMBO J 1996;15:3377-3384.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01611-02

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The VHL Tumor Suppressor Gene

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard D. Klausner, M.D.	Chief, CBMB, NICHD	
	D. Chen	HHMI	CBMB, NICHD
	J. Humphrey	Adjunct Scientist	CBMB, NICHD
	S. Lee	Visiting Fellow	CBMB, NICHD
	A. Pause	Visiting Fellow	CBMB, NICHD

COOPERATING UNITS *(if any)*

Urologic Oncology Section, Surgery Branch, DCS, NCI (W. Marston Linehan)

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Section on Organelle Receptor Structure and Function

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.8	4.0	.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The VHL gene was first identified in 1993 as the gene responsible for a rare inherited cancer syndrome called Von Hippel-Lindau disease. Inheritance of a single defective VHL gene results in the predisposition to the development of a variety of cancers including clear cell renal carcinoma, pheochromocytoma, and CNS hemangioblastoma. Subsequently, it was shown that the majority of tumors derived from patients with sporadic clear cell renal carcinoma also contained both loss of heterozygosity of the VHL locus and failure of expression and/or mutations in the remaining VHL allele. The goal of this project is to understand the biochemistry and cell biology of the VHL gene product and thereby to elucidate its role in cell transformation. Our previous studies include the completion of the cloning and sequencing of the human and rat VHL gene, the biochemical characterization of the gene product and its intracellular localization. The identification and cloning of gene products that VHL interacts with has illuminated an unexpected new area of gene control--the regulation of transcriptional elongation. We have continued our characterization of the interaction between the VHL proteins and other cellular components. In order to identify new potential interacting proteins we utilized recombinant VHL-Elongin B/C complexes to affinity purify additional cellular interacting proteins. One of the proteins has been recently identified as a homolog of the new recognized Cullin family of proteins. Two other interacting proteins have been identified by far Western blot analysis which we are now attempting to clone by an expression approach. We have shown that VHL regulates the expression of vascular endothelial growth factor (VEGF) in a post-transcriptional manner. We are currently performing studies to identify the VEGF 3'UTR cis-element responsible for VHL-mediated regulation. We have extensively studied transport of the VHL protein and shown cell density and such factors as low serum conditions are critical to transport of VHL into and out of the nucleus. We have developed a knockout mouse model to determine VHL function in the mouse. Embryos homozygous for this mutation die in utero between 7 and 8.5 days. Ongoing studies suggest a failure of blood-island formation and vasculogenesis in the labyrinth of the yolk-sac.

THE VHL TUMOR SUPPRESSOR GENE

Project Description

Major Findings

Biochemistry of the VHL Protein

We have continued our characterization of the interaction between the VHL protein and other cellular components. Studies with stable cell lines expressing epitope-tagged VHL strongly support the notion that VHL exists as a stable heterotrimer bound tightly to one molecule each of Elongin B and Elongin C. The majority of naturally occurring exon 3 mutations map to the Elongin interaction site previously defined and no other mutations or truncations seem to affect binding with the BC subunits. In order to identify other potential interacting proteins, we utilized recombinant VBC epitope-tagged complexes in order to affinity purify additional cellular interactors. Such an approach produced a series of proteins that were specifically bound to the VBC complex. Extensive biochemical studies convinced us that a set of proteins between 70 and 80 kD were specifically bound to VBC and were part of an endogenous cellular complex. These proteins were purified and peptide sequences were obtained. One of the proteins has recently been identified as a homolog of a newly recognized family proteins called cullins. CUL-1 was first reported in June 1996, as a C elegans gene that is required to maintain differentiated cells in the G_0 state. Sequence homology suggested at least six human homologs to CUL-1 in the database. These are referred to as CUL-1 through 6. The 76 kD VBC interacting protein that we identified is human cul-2. The ability to bind cul-2 is restricted to VBC complexes and preliminary data suggests that alterations in exon 1 or 2 of VHL may interrupt this interaction, in contrast to the interaction with B and C. The cullins are homologous to the *S. cerevisiae* CDC53 gene product which is also involved in cell cycle regulation and a recent report strongly suggests that CDC53 encodes an E3 ubiquitin conjugating enzyme.

An additional approach to the identification of VBC interacting proteins is to utilize phosphorylated VBC after modification of the VHL protein with a protein kinase phosphorylation site sequence and to use the phosphorylated VBC complex as a probe in a far Western blot of proteins derived from cell lines. Such an approach has identified two other strongly interacting proteins which we are now attempting to clone by an expression approach.

VHL and the Regulation of Gene Expression

In order to understand how the VHL gene product works in cells, we sought to identify potential target genes that might be regulated by VHL expression. We had demonstrated that when human kidney cancer cell lines were assayed for tumor genicity in nude mice, tumorogenic potential could be inhibited by stable retroviral transduction of the cell lines with wild-type, but not mutant, VHL. In looking at the histology of the resulting tumors, we were struck by the lack of vasculature and cellular infiltrate in the small tumor that arose from the VHL wild-type expressing cells. These cells grow normally in culture and the disparity between *in vitro* cell growth and tumor formation suggested that VHL was regulating genes that relate to the ability of the tumor cells to spread, invade or metastasize. When the tumors were stained for an angiogenic factor called vascular endothelial growth factor (VEGF), we noticed that the parental tumor line or the tumor line expressing mutant VHL made easily detectable VEGF protein. In contrast, the cell line transduced with wild-type VHL made no detectable VEGF protein. Supernatants of these different cell lines were used to test for the ability to stimulate proliferation of bovine endothelial capillary cells. Mitogenic activity was found for all the cells lines but was consistently significantly higher in the parental and mutant VHL tumor lines than in the wild-type transduced line. All of the additional mitogenic activity for these vascular endothelial cells could be inhibited by neutralizing antibodies against VEGF. We next demonstrated that the wild-type cell lines had very low levels of VEGF mRNA compared to the parental line or a line transduced with a mutant VHL. We looked to see whether this difference in the level of VEGF mRNA could be ascribed to altered rates of transcription or transcription elongation. For this purpose nuclear run-ons were performed using probes from different regions of the transcript. Despite significant differences in VEGF mRNA levels, there was no difference in the rate of transcription or in the profile of transcription activity across the transcript, strongly suggesting that VEGF mRNA control is not due to either transcription or transcription elongation but rather is most likely due to the regulation of mRNA half-life. We additionally noted that the diminution of VEGF mRNA levels in the cells expressing wild-type VHL was conditional. When the cells were stressed by being grown in low serum conditions, VEGF mRNA levels rose to that equal to those seen in the parental cell line. In other words, the presence of VHL in the cell allows the cell to regulate VEGF mRNA levels, most likely at the level of mRNA stability, in response to extracellular signals.

Cell Biology of the VHL Gene Product

Our initial studies on the localization of epitope-tagged VHL in a variety of cells demonstrated a puzzling pattern in which some cells had predominantly nuclear and others cells predominantly cytoplasmic VHL localization. We have extensively studied this phenomenon. Examination of a variety of cell growth conditions suggested that confluency profoundly affected the distribution of VHL as determined by indirect immunofluorescence microscopy. When cells were grown to confluent conditions, the distribution of VHL was largely cytoplasmic with varying degrees of nuclear exclusion. In contrast, in cells grown to very sparse conditions, the VHL protein was largely confined to the nucleus. Even with indirect immunofluorescence, this was not an all or none effect. Mutational analysis of the VHL protein demonstrated that sequences in the first exon were required for nuclear localization. This region contains a classic bipartite basic nuclear localization signal. Exon 3 mutations, particularly those that result in loss of interaction with Elongin B and C resulted in predominantly nuclear staining, regardless of the cell growth conditions. These findings became easier to interpret by looking at the distribution of VHL protein in living cells. This was accomplished by making fusions between the VHL protein and the green fluorescent protein (GFP). We demonstrated that the VHL-GFP fusion protein bound to Elongin B and C in a manner identical to that seen for the wild-type VHL. Furthermore, VHL-GFP reproduced the ability of VHL to regulate VEGF mRNA expression strongly suggesting that the fusion protein was functional and normal. Examination of the distribution of VHL-GFP in cells in culture under different growth conditions gave us somewhat different results than the indirect immunofluorescence. While confluent cells had more cytoplasmic than nuclear staining, sparse cells showed a significant amount of cytoplasmic staining, much more than had been seen by indirect immunofluorescence. The indirect immunofluorescence results could be reproduced by treating the cells expressing VHL-GFP under the same conditions used to prepare cells for immunofluorescence. This included cross-linking with formaldehyde and permeabilization with saponin. The cross-linking resulted in no effect on the distribution of VHL-GFP. However, when the cross-linked cells were permeabilized, the cytoplasmic staining seen in sparse cells was almost entirely lost, leaving only a nuclear pattern. This was not seen in confluent cells. This effect was quantitated using a CCD camera. Thus, cells in sparse conditions contained VHL in both the nucleus and the cytosol, but cytosolic VHL could not be cross-linked to other cellular components. Cytosol VHL could be cross-linked in confluent cells. Elongin box VHL mutants could not be cross-linked and stabilized in the cytosol under any growth conditions, suggesting that the VBC interaction was essential for cytoplasmic cross-linking. Noting that under sparse conditions VHL-GFP could not be fixed in the cytosol led us to ask whether VHL was cycling between the nucleus and the cytosol. To test this, we used a variety of drugs hoping that one or more would block either nuclear import or nuclear export and result in a shift in the distribution of the VHL protein. Such an effect was seen with the transcriptional inhibitor actinomycin D. For VHL that cannot be cross-linked and stabilized in the cytosol, the addition of actinomycin D resulted in a rapid redistribution of VHL from cytosol to the nucleus. This was particularly rapid for mutant VHL that cannot bind Elongin B/C, but was also seen for wild-type VHL, although at a slower rate, and only under sparse conditions. In contrast, wild-type VHL in confluent cells shows no redistribution of VHL over two hours in the presence of actinomycin D. This effect could not be duplicated with inhibitors of protein synthesis. This suggested a model whereby VHL cycles between the cytosol and the nucleus requiring ongoing transcription in order to be exported from the nucleus. One step in the cycle that appears to be regulated by environmental signals, in this case confluency, is retention in the cytosol which can be measured both by the failure to redistribute the presence of actinomycin D and the ability to cross-link the VHL in the cytosol to undefined cellular components.

We sought to correlate the distribution and cycling of VHL with the regulation of VEGF mRNA levels. We asked whether cells expressing a mutant or wild-type VHL demonstrated altered VEGF mRNA as a function of confluency, under the same conditions used to observe the redistribution of the VHL protein. We found that in a variety of kidney cancer cell lines, VEGF mRNA levels rose dramatically when cells were grown in confluent conditions when compared to sparse conditions. This was only seen in cells failing to express VHL or expressing mutant VHL. In cells expressing wild-type VHL, VEGF mRNA levels did not change as a function of confluency. It appears from these results that confluency, independent of VHL, regulates VEGF mRNA levels. At the same time, confluency results in some alteration that affects the distribution of VHL resulting in it being stably in the cytoplasm interacting with other cellular components. This simultaneous affect on VHL distribution and VEGF mRNA by confluency cancel each other and wild-type VHL abrogates the ability to induce VEGF mRNA levels in response to confluency. These results strongly suggest that VEGF mRNA levels, which are regulated by VHL at the level of mRNA stability, requires VHL to be in the cytosol.

VHL Function in the Mouse

In order to determine the effect of heterozygous or homozygous deletion of VHL in a mammal, knock-out mice were generated by standard techniques. Heterozygous mice develop normally and out to one year we have seen no evidence of tumors. In contrast, homozygous knock-out of VHL is an embryonic lethal event. We have recently attempted to pinpoint the point in embryonic development at which this lethality is seen. It appears to be very early between embryonic day 7.5 and 8.5 and preceeds organogenesis. The most striking abnormality is the failure to develop a structure called the labyrinth, a vascular structure elaborated by the embryo that allows the intermixing of maternal and fetal blood across endothelial barriers. In the absence of VHL, there is essentially no significant development of the vasculature in this organ and no fetal nucleated red cells present. This region of the fetus demonstrates hemorrhage resulting from the extrusion of maternal cells and presumably is responsible for the early and uniform loss of the embryos.

Publications:

Gnarra JR, Duan DR, Weng Y, Humphrey JS, Chen DYT, Lee S, Pause A, Dudley CF, Latif F, Kuzmin I, Schmidt L, Duh FM, Lerman MI, Zbar B, Klausner RD, Linehan WM. Molecular cloning of the von Hippel-Lindau tumor suppressor gene and its role in renal carcinoma. Biochim Biophys Acta 1996;1242:201-210.

Gnarra J, Zhou S, Merrill MJ, Wagner J, Krumm A, Papavassiliou E, Oldfield E, Klausner RD, Linehan WM. Post-transcriptional regulation of vascular endothelial growth factor mRNA by the VHL tumor suppressor gene product. PNAS USA 1996;in press.

Humphrey J, Klausner RD, Linehan WM. Von Hipple-Lindau syndrome: hereditary cancer arising from inherited mutations of the VHL tumor suppressor gene. In: H.J. Pienta (ed.), Diagnostic advances: the use of molecular medicine in the diagnosis and prognosis of genitourinary malignancies. Boston: Khuwer, 1996;in press.

Lee S, Chen DYT, Humphrey JS, Gnarra JR, Linehan WM, Klausner RD. Nuclear/cytoplasmic localization of the VHL tumor suppressor gene product is determined by cell density. PNAS USA 1996;93:1770-1775.

Pause A, Aso T, Linehan WM, Conaway JW, Conaway RC, Klausner RD. Analysis of the interaction of the VHL tumor suppressor gene product with Elongin. Methods in Enzymology 1996;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1-HD-01612- 01

PERIOD COVERED

July 1, 1996 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic Analysis of RNA Polymerase II Transcriptional Regulation in Drosophila

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.A. Wassarman	Senior Staff Fellow	CBMB, NICHD
Others:	Q. Phan	Pre-IRTA	CBMB, NICHD

COOPERATING UNITS *(if any)*

Department of Molecular and Cell Biology, University of California at Berkeley (F. Sauer and R. Tjian).

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Unit on Developmental Transcriptional Regulation

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Transcription of protein coding genes by RNA polymerase II is directed by a multiprotein complex that includes gene-specific enhancer binding factors and basal transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH). In vitro, basal transcription factors are sufficient to direct a low level of transcription from a core promoter, but enhancer binding factors and their DNA binding sites are required to achieve a higher level of transcription, termed activated or regulated transcription. Protein-protein interactions between the activation domains of enhancer binding factors and one or more subunits of TFIID mediate activated transcription in vitro. TFIID is a multiprotein complex composed of TATA-binding protein (TBP) and at least eight TBP-associated factors (TAFs). Specific TAFs bind different classes of activators (such as acidic, glutamine rich, or isoleucine rich). In addition, TAFs contact other components of the transcriptional machinery, recognize and contact specific DNA sequences in core promoters, and have enzymatic activities that may contribute to gene-specific transcriptional activation in vivo. We have isolated mutations in several Drosophila TAFs in a screen for genes that dominantly modify a rough eye phenotype caused by a transgene expressing a gain-of-function form of the Ras1 GTPase. TAF mutations reduce the level of transcription of the Ras1 transgene as well as transcription from other, but not all, RNA polymerase II genes. Drosophila was originally chosen as the model organism to study the role of TFIID and TAFs in transcriptional regulation because it is amenable to biochemical, molecular, and genetic approaches, and it displays complex developmental and tissue-specific transcriptional regulation. The establishment of this sensitized genetic system in Drosophila presents the opportunity to probe in vivo roles for TAFs and, in combination with the wealth of biochemical data on TAFs, to decipher the molecular mechanisms controlling transcriptional activation.

ZO1 HD 01612-01

GENETIC ANALYSIS OF RNA POLYMERASE II TRANSCRIPTIONAL FACTOR IN DROSOPHILA

Project Description

Objective:

To understand the mechanisms controlling transcriptional activation of RNA polymerase II genes. As of the writing of this report we are in the process of setting up the laboratory and therefore have no new findings to present at this time. Below are projects that will be undertaken in the coming year.

Proposed Course of Project:

1. Genetic screens for transcription factors: Using sensitized genetic backgrounds that affect Drosophila eye development, we will conduct genetic screens to isolate mutations in known components of the RNA polymerase II transcriptional machinery, as well as identify general and gene-specific components of this multiprotein complex. The isolation of mutations in this class of genes will provide important tools for studying transcriptional regulation during Drosophila development.

2. Analysis of promoter utilization and recognition by TAFs: Mutations in TAFs suppress rough eye phenotypes caused by genes expressed under control of the sevenless (sev) enhancer and promoter but not the sev enhancer and heat shock protein 70 (hsp70) promoter. Using the rough eye phenotype as a reporter for transcription level in vivo, the sev, hsp70, and other promoters will be mutagenized and assayed for altered phenotypes in TAF mutant flies. In this manner, it may be possible to define promoter sequences and specific TAFs that are involved in promoter discrimination.

3. Characterization of sev transcription factors: A number of the screens described above rely on sev regulatory sequences to express genes in the Drosophila eye. To understand how TAFs are functioning in this context, it is important to identify factors and DNA sequences that regulate sev transcription. Previous studies have defined a 475-bp enhancer fragment that confers correct qualitative and quantitative expression of the sev gene. Enhancer binding factors that recognize this fragment and activate transcription will be identified by molecular methods. In addition, mutations in these genes may be isolated in the genetic screens.

4. Biochemical analysis of mutant transcription factors: Mutant proteins isolated in the screens described above will be assayed in vitro to determine how they affect transcriptional activation.

Significance to Biomedical Research and the Program of the Institute:

Transcriptional regulation is a basic process that occurs in all eukaryotic organisms. Mutations that affect the level, timing, or spacial distribution of transcription can cause severe developmental abnormalities. Since the transcriptional machinery displays a high degree of evolutionary conservation, an understanding of the factors and mechanisms that regulate transcription in Drosophila should be easily extrapolated to humans.

DEB

NICHD Annual Report
October 1, 1995 to September 30, 1996

Developmental Endocrinology Branch

The DEB investigates fundamental issues in human reproduction, growth and development. Researchers within this branch employ genetic, biochemical and physiological analyses to elucidate the molecular mechanisms underlying the wide array of clinical disorders available for study in our reproductive, pediatric and adult endocrinology clinics. Current projects focus on the role of sex steroids and growth factors in regulating statural growth and reproductive function; the molecular biology of steroid and glycoprotein hormones and their receptors; the role of CRH, vasopressin and glucocorticoids in stress and immune responses; mechanisms of ovarian follicular failure in premature and normal menopause and the genetic analysis of multiple endocrine tumor syndromes and syndromes associated with alterations in genomic imprinting. The wide range of interests and patient populations within the DEB creates a challenging, intellectually stimulating environment conducive to fruitful collaborations and scientific exchanges and supports vigorous training programs in pediatric, reproductive and adult endocrinology.

The Section on Growth and Metabolism, led by Carolyn Bondy, investigates mechanisms of growth regulation, focusing on the actions of insulin-like growth factors-I and -II (IGFs-I and -II). These growth factors have major roles in development as proven by the fact that targeted deletion of IGF-I and IGF-II gene expression results in severe dwarfism and, in the case of IGF-I, infertility in both sexes— although how IGF deletion causes these defects is not yet understood. During the past year this Section has investigated the molecular mechanisms of IGF action in regulating growth and reproduction by utilizing these IGF 'knockout' mice models.

IGF-I and the Nervous System. The highly metabolically active brain relies almost exclusively on glucose to supply fuel. Despite the fact that the brain constitutes only about 2% of body weight, it accounts for over 20% of the body's glucose consumption. The manner in which brain glucose metabolism is regulated, however, is poorly understood. Dr. Bondy's group has shown that little if any insulin is synthesized within the brain and very little crosses the blood brain barrier. They have also demonstrated that neuroanatomical patterns of IGF-I and IGF-I receptor expression closely parallel regional brain glucose utilization. Based on these findings, they hypothesized that locally produced IGF-I might serve to regulate neuronal glucose metabolism.

To evaluate this hypothesis, the expression of proteins involved in glucose transport and metabolism was compared in brains from targeted IGF-I gene deletion and wild type early postnatal and adult mice using western blotting. The neuron-specific glucose transporter, GLUT3, and the blood brain barrier glucose transporter, GLUT1, are increased in the IGF-I null brain. Hexokinase, the rate limiting enzyme in neuronal glucose utilization, is reduced to less than one third that found in wildtype brain. In contrast, enzymes involved in the glycolytic generation of ATP (which are negatively regulated by ATP level) such as pyruvate kinase and neuron specific enolase, are up-regulated in IGF-I knockout brains. Interestingly, CPK, an ATP-generating enzyme which is characteristically expressed in hypoxic or ischemic states, is also increased in knock out brains. Values for heterozygote brains fall midway between wildtype and homozygous deletion brains in all these cases. In vivo studies of regional brain glucose utilization employing the 14C-2-deoxyglucose technique reveal a selective reduction in glucose uptake in the sensory projection centers (e.g., olfactory bulb and inferior colliculus - which express the highest IGF-I levels in normal adult brain). In vitro studies showed that glucose uptake is significantly decreased in synaptosomes from knockout brains and normalized by addition of IGF-I. Additional studies demonstrated that IGF-I treatment of fresh synaptosomal preparations induces fusion of endosomes with the nerve terminal membrane and consequent translocation of GLUT3 to the synaptic aurface, along with a number of specific endosomal-specific proteins including dynamin and synaptotagmin.

These data support a fundamental role for local IGF-I production in regulation of brain glucose metabolism. By analogy with the known effect of insulin on glucose transporter function in the periphery, it is possible that GLUT3 shuttling from intracellular vesicle to neuronal plasma membrane surface is impaired in the absence of IGF-I. Alternatively, the defect in neuronal glucose uptake may be due primarily to the reduction in hexokinase expression in IGF-I knockout brains. The decrease in neuronal glucose transport and phosphorylation is predicted to result in diminished ATP production and compensatory up-regulation of ATP sensitive enzymes.

IGFs and the Reproductive System. The group has previously reported that IGF-I expression is restricted to granulosa cells in a subset of preovulatory follicles in the rodent ovary and shown that IGF-I positive follicles are characterized by cell proliferation and FSH receptor (FSHR) expression. Recent work showed that FSHR is undetectable in the IGF-I null mouse ovary, suggesting that follicular IGF-I regulates granulosa cell FSHR expression and hence determines follicular growth and maturation in response to gonadotropin stimulation. This prediction has been confirmed in dynamic studies of the IGF-I knockout mouse showing that the IGF-I null female is gonadotropin resistant, remaining prepubertal despite a normal pubertal rise in pituitary gonadotropins, and demonstrating relative resistance to ovulation induction by pharmacological treatment with gonadotropins.

This section's work has previously shown that human and rhesus monkey ovaries demonstrate a similar pattern of IGF system expression and that the primate pattern diverges very significantly from the rodent, such that studies in the mouse may not be directly relevant to the human. Thus the group has pursued studies of the IGF system in rhesus ovaries. They have now shown that a specific IGF binding protein, IGFBP4, is selectively expressed in dominant follicles in the rhesus and also in the human ovary. The spatiotemporal patterns of IGFBP4 expression parallel LH receptor expression and granulosa cell luteinization, suggesting IGFBP4 expression is regulated by LH and that attenuation of IGF effect by this inhibitory binding protein regulates the progression of follicular development from the proliferative, estradiol-synthetic phase to the differentiated, progesterone-synthetic stage.

One of the fundamental differences between murine and primate ovary IGF systems is that murine follicles selectively express IGF-I while primate follicles constitutively express IGF-II.

The group hypothesized that the different patterns of IGF expression are related to the radically different ovarian cycles demonstrated by these species, with mice producing 10-15 ovulations every 4 days while primates produce one every month. To investigate this hypothesis, patterns of IGF system expression were examined in the porcine ovary, which represents a composite of the above patterns, with 10 or more ovulatory follicles produced every three weeks. Interestingly, both IGFs were detected in porcine follicles, with IGF-I displaying a selective and IGF-II a constitutive pattern of expression, suggesting that IGF-II expression is associated with the prolonged process of follicular development seen in larger species while IGF-I expression is associated with the development of multiple follicles each cycle.

Growth Hormone Stimulates Primate Mammary Tissue Hyperplasia. Since declining GH levels in aging individuals have been correlated with decreases in lean body mass, particularly in the musculoskeletal system, GH 'replacement therapy' has recently been advocated for aging. In a recent study of elderly female rhesus monkeys treated with GH for 7 weeks, Bondy and colleagues noted the GH-treated monkeys to have mammary enlargement relative to saline treated animals. Histologic evaluation of the mammary tissue revealed a highly significant increase in epithelial cell proliferation rate as determined by expression of proliferation specific antigens. The proliferative effect was positively correlated with circulating GH but not circulating estradiol or progesterone levels. There was a weak positive correlation with circulating IGF-I levels and no changes in local, mammary IGF-I production. These observations suggest that caution is necessary in the use of GH treatment in aging women at risk for breast cancer.

Section on Women's Health

Ovarian function. A major focus of this group for the past few years has been premature ovarian failure which affects approximately 1% of women. Dr. Lawrence Nelson has accumulated the largest series of patients with premature ovarian ever studied prospectively and has characterized this group using state of the art imaging, immunological and molecular analyses. Extensive and exhaustive testing has revealed that only a minority of these women have evidence of autoimmune ovarian failure, as demonstrated by rigorous screening for circulating anti-ovary antibodies and negative ovarian biopsies. These findings have major therapeutic implications, since at present many women with premature ovarian failure are empirically treated with risky immunosupressive therapy, which our data would suggest is rarely justified. Furthermore, in contrast to the commonly held view that premature ovarian failure is due to irreversible oocyte depletion, Nelson and colleagues have found that almost half the affected women have ovarian follicular development detectable by ultrasound and by rising serum estradiol levels. Biopsy of these follicles has shown a consistent pattern of premature luteinization, suggesting the presence of a functional and potentially reversible disorder of folliculogenesis. Current studies are aimed at elucidation of the functional deficit with a view to designing novel therapeutic approaches to the loss of ovarian function which is so devastating to these young women.

Obesity. Obesity affects approximately one third of the female population in the United States and is even more prevalent among African-American women. Since African-American women are at substantially greater risk for developing complications of obesity including hypertension, diabetes mellitus, nephropathy, retinopathy, vascular disease and death compared with Caucasian women, the elucidation of differences in pathophysiological mechanisms related to obesity in these populations is of great medical consequence. Substantial evidence suggests that risk factors and therapeutic approaches derived from the study of Caucasians may not be accurate or effective when applied to individuals of differing ethnicity or race. Dr. Jack Yanovski's clinical studies have demonstrated that significant differences are found in anatomical patterns of fat deposition, insulin sensitivity and basal metabolic rate in African-American and Caucasian women and girls. For example, it was found that visceral adiposity is relatively lower in African-American females and that insulin resistance is correlated to the amount of subcutaneous but not visceral adipose tissue. These findings indicate that the risk factors identified in studies of predominantly Caucasian populations may not be relevant for other genetic groups, and may not, in fact, represent true pathogenetic factors. Current studies are directed at evaluating the genetic, physiological, metabolic and behavioral factors involved in the ontogeny of obesity in children of both races.

Nuclear hormone receptors. A group led by Dr. James Segars carries out basic studies investigating modulators of retinoic acid receptor and estrogen receptor action in reproductive tissues. Using an interactive cloning strategy to isolate gene products from breast cancer cells capable of binding to nuclear receptors, they have identified a novel hormone receptor binding protein, denoted Brx-1, which is expressed in reproductive tissues and enhances estrogen receptor action. Current studies are directed at determining the physiological role of Brx-1 and related proteins in reproductive function.

Endometrial physiology. The Unit on Reproductive Medicine, led by Dr. Lynnette Nieman, has focused in recent years on the investigation of factors regulating endometrial receptivity. Progesterone is essential for the development of a functional secretory endometrium. Abnormal endometrial development, whether because of inadequate progesterone secretion or because of inadequate endometrial responsiveness, may underlie infertility and multiple abortion syndromes. Clinical studies by Dr. Nieman's group employ anti-progesterone pharmacological agents to elucidate cellular and biochemical mechanisms of progesterone action on the luteal phase endometrium.

Menopause. Dr. Bondy and others in the Section on Women's Health have developed a non-human primate model for the elucidation of tissue specific effects of the major menopausal hormone replacement therapy regimens. Ovariectomized rhesus monkeys are treated with subcutaneous implants containing estradiol (E2) alone, E2 plus progesterone (P4), E2 plus testosterone, tamoxifen or saline. Uterine, vascular, mammary and other hormone sensitive tissues are evaluated for changes in expression of growth factors and their receptors as well as for markers of cellular proliferation and differentiation. In the first data from this study, the effects of sex steroids on the IGF system in the primate myometrium were analyzed. IGF-I and IGF-I receptor mRNAs are co-expressed by smooth muscle cells, supporting the possibility for autocrine/paracrine IGF action in stimulating myometrial growth. IGF-I mRNA is barely detected in control myometrium, is significantly increased by E2 treatment and is augmented even more by combination E2&P4 treatment, while little change is noted in myometrial IGF-I receptor mRNA levels. The Ki-67 proliferation-specific antigen positive myometrial nuclei are also significantly increased by E2 and are augmented even more by E2&P4 treatment, with a highly significant positive correlation between local IGF-I mRNA concentration and local Ki-67 positive cell count. These data provide the first direct experimental evidence for regulation of IGF-I gene expression by sex steroids in the primate uterus and implicate local IGF-I action in both estrogen- and progesterone-induced myometrial growth. The finding that P4 adds very substantially to E2's growth stimulating effects on myometrial tissue has important clinical implications, since P4 given to menopausal women to 'protect' the endometrium may serve as a double-edged sword putting the myometrium at risk.

The Unit on Genetics and Hormone Action, led by Domenico Accili, conducts studies of the genetic basis of non-insulin-dependent diabetes mellitus (NIDDM). NIDDM is currently viewed as a complex metabolic disorder with multiple causes. The metabolic hallmarks of NIDDM are peripheral insulin resistance and impaired insulin secretion. The two defects are intimately intertwined in most NIDDM patients. However, in longitudinal studies of patients at high risk of developing NIDDM, it appears that insulin resistance is the first identifiable metabolic abnormality. Insulin resistance is a genetically complex trait, i.e., it is not inherited in a Mendelian fashion and simple genetic models, such as studies of candidate genes, have failed to identify the gene(s) responsible for insulin resistance. Several aspects of insulin action appear to be impaired in NIDDM but the nature of the fundamental defect remains unclear. It is widely held that a defect at an early post-receptor step in insulin action could provide a unifying mechanism for insulin resistance. In recent years, the pace of discovery of new molecules and novel signaling mechanisms downstream of the insulin receptor (IR) has quickened considerably. However, studies of genes encoding proteins that participate in the insulin signaling systems have failed to implicate one or more of these genes as a major candidate for the ethiology of NIDDM. It is possible that, because of genetic heterogeneity, different genes are responsible for insulin resistance in different patients. Furthermore, the functional impairment caused by some genetic variations might be too subtle to be detected by the assays at our disposal, or may require the combined interactions of other mutant genes to give rise to the diabetic phenotype. Using mouse and human genetic models, Accili and coworkers are trying to dissect the genetic components of insulin resistance.

Targeted Mouse Mutants with Insulin Resistance and Diabetes. This project focuses on the characterization of mice with targeted mutations in the pathway of insulin action. To generate a mouse model of insulin resistance, scientists in the Unit have initially introduced a mutant allele of the insulin receptor in mice. Mice born without functional insulin receptors have normal features at birth; their intrauterine growth and development appear to be normal. However, upon feeding they develop dramatic metabolic alterations with hyperglycemia and hyperketonemia. Within few days, mutant mice die of diabetic ketoacidosis (DKA). Thus, lack of insulin receptors in mice impairs fuel metabolism. The early post-natal death caused by inactivation of the insulin receptor gene is reminiscent of other targeted inactivations of genes important for fuel metabolism, such as glucokinase and C/EBP-α.

A Mouse Model of Non-Insulin-Dependent Diabetes Mellitus, the NIDDM mouse. An important contribution of the Unit has been the development, in collaboration with Dr. C.R.Kahn's laboratory at the Joslin Diabetes Center, of a polygenic mouse model of NIDDM.

In these experiments, mice heterozygous for an insulin receptor null allele have been crossed with mice heterozygous for a null mutation of the gene encoding IRS-1, a major substrate of the insulin receptor kinase. IRS-1 is thought to mediate insulin and IGF-1 signaling by way of interactions with adaptor molecules such as phosphatidylinositol (PI)-3-kinase, grb-2, sos, syp (tyrosine phosphatase 1B), and many others. Mice lacking IRS-1 are growth retarded and mildly insulin resistant, consistent with a model in which IRS-1 mediates both the actions of insulin and IGF-1 receptors. Mice heterozygous for both an insulin receptor and an IRS-1 mutant allele develop all the hallmarks of insulin resistance: hyperinsulinemia and hyperplasia of pancreatic beta cells. Within two months of birth, they become frankly hyperglycemic, thus resembling very closely the pathogenesis of human diabetes. These findings are consistent with a polygenic mode of inheritance of NIDDM. This mouse model of NIDDM represents a significant advance over existing animal models of this condition, such as the ob/ob and db/db mice, which are primarily mouse models of obesity. In a broader context, lessons learned from this mouse model have a substantial impact on current gene mapping strategies in common diseases, because they demonstrate how two genes can interact epistatically in the pathogenesis of these disorders.

Insulin Receptors in Embryonic Development. Insulin receptors are expressed in the pre-implantation mouse embryo, and insulin has been shown to stimulate glucose utilization by isolated blastocysts. It is therefore somewhat surprising that embryonic growth and development are not affected by the lack of insulin receptors. The role of insulin receptors in fetal life is a matter of debate. In addition to the growth retardation observed in cases of extreme insulin resistance, it should be remembered that macrosomia is a well recognized complication of fetal hyperinsulinemia, a metabolic consequence of the diabetic pregnancy. The phenotype of nullizygous insulin receptor mice suggests that, during gestation, insulin receptors can be replaced by other receptors. Because of the close structural and functional homology between insulin and IGF-1 receptors, the IGF-1 receptor is a prime candidate to compensate for functions normally exerted by insulin receptors. This question has been examined in more detail in collaboration with Dr. A. Efstratiadis's laboratory at Columbia University. Dr. Efstratiadis has shown that mice lacking IGF-1 receptors are severely growth retarded and die of unexplained complications shortly after birth. Likewise, mice lacking IGF-2 receptors die immediately after birth, but their body weight is increased by ~30%. Surprisingly, mice lacking both IGF-1 and IGF-2 receptors survive and have normal size. We have tested the hypothesis that the insulin receptor is the receptor responsible for survival of mice lacking both the IGF-1 and IGF-2 receptors. Mice lacking insulin as well as IGF-1 and IGF-2 receptors have been derived. Indeed, mice lacking both the insulin and the IGF-1 receptor are smaller than mice lacking either receptor alone (30% vs 45% for mice lacking IGF-1 receptors and 100% for mice lacking insulin receptors). Mice lacking all three receptors (insulin, IGF-1 and IGF-2) are even smaller than the double insulin/IGF-1 knock-out mice. These data indicate that insulin receptors play an ancillary role in embryonic growth. It is likely that these actions of the insulin receptor are mediated in response to IGF-2 binding, since the knock-outs of the two ligands, insulin and IGF-1, suggest that the actions of these two hormones are only mediated by the respective receptors. It remains to be determined whether the growth-promoting actions of the insulin receptor are mediated by holodimeric insulin receptors (composed of two α- and two β-subunits) or by heterodimeric receptors composed by an insulin receptor $\alpha/\ \beta$ monomer and an IGF-1 receptor $\alpha/\ \beta$ monomer. Experiments to address this question are underway.

The Unit on Growth and Development, led by Jeffrey Baron, investigates the cellular and molecular mechanisms governing bone growth and mineral metabolism. This knowledge is applied to improve medical treatment in disorders of bone growth and in disorders of mineral metabolism. Through the study of longitudinal bone growth, the investigators also seek to uncover general principles of developmental biology since the cellular processes underlying bone growth, such as cell proliferation, terminal differentiation, angiogenesis, and cell migration, are essential for development in other tissues.

Fibroblast Growth Factors (FGFs) and FGF Receptors. Fibroblast growth factors (FGFs) and FGF receptors are expressed by growth plate chondrocytes. Addition of FGFs to growth place chondrocyte culture stimulates the anabolic processes underlying chondrogenesis and inhibits terminal (hypertrophic) differentiation. In humans, mutations in FGF receptors cause skeletal dysplasias, thus confirming the importance of these growth factors in bone growth. The investigators showed that an in vivo infusion of FGF-2 into the growth plate accelerated local vascular invasion and ossification of growth plate cartilage. These results suggest the possibility that FGF endogenous to the growth plate attracts the normal invasion of vessels and bone cells from adjacent metaphyseal bone. These investigators are collaborating with workers in the National Institute for Genome Research to identify and characterize genetic defects in FGF or FGR receptors responsible for deficient long bone growth in a spectrum of children with "idiopathic" short stature.

Glucocorticoid Effects. In children, glucocorticoid therapy provides effective treatment for many disorders but can severely inhibit linear growth. Investigating the underlying mechanism, Dr. Baron and coworkers demonstrated that an in vivo infusion of glucocorticoid directly into growth plate significantly inhibited longitudinal bone growth. These data indicate that excess glucocorticoid causes a rapid potent inhibition of growth by a direct local action on the growth plate. Subsequent studies explored the hypothesis that this local action involves interference with the expression of growth hormone receptor. Contrary to this hypothesis, however, glucocorticoid administration significantly increased GH receptor mRNA levels in growth plate.

Ca^{2+}-Sensing Receptor Mutations. This research group identified Ca^{2+}-sensing receptor (CaR) gene mutations in four families with autosomal dominant hypoparathyroidism. All were heterozygous missense mutations. Because the CaR inhibits parathyroid hormone secretion, these mutations must activate the CaR. The resulting phenotype varies from asymptomatic to life-threatening, depending on the specific mutation. The investigators also demonstrated that de novo mutations in this gene cause sporadic hypoparathyroidism. Identification of these mutations in sporadic cases defines the risk for the patient's future offspring, thus allowing appropriate genetic counseling.

Since the CaR also inhibits calcium reabsorption from the glomerular filtrate, the investigators predicted that activating CaR mutations would cause hypercalciuria even at low serum calcium concentrations. They empirically confirmed this prediction by comparing calcium excretion in patients with activating mutations to patients with other forms of hypoparathyroidism. This finding has an important clinical implication; patients with activating CaR mutations are at greater risk for hypercalciuria and its adverse consequences than are other hypoparathyroid patients and thus they may benefit from treatments that lower urinary calcium excretion.

The Section on Developmental Endocrinology, led by Gordon Cutler, investigates the basic mechanisms and clinical disorders of growth, puberty, and adrenal function. The objective is to gain new insights into molecular mechanisms, physiology, and pathophysiology, which can be applied to the prevention, diagnosis, and treatment of endocrine diseases. The methods employed range from those of molecular endocrinology to controlled clinical trials.

New Approaches to the Treatment of Short Stature. Four major clinical trials are in progress that are designed to evaluate new approaches to the treatment of short stature. The first tests the hypothesis that prolonging the growth period by delaying puberty can enhance adult height. An interim analysis indicates that delay of puberty by an average of 3.6 years increases adult height by 6.8 cm compared to the randomized, placebo-treated control. The second clinical trial tests the hypothesis that supplemental growth hormone (GH), with or without low-dose ethinyl estradiol, will increase the adult height of girls with Turner syndrome. Since girls with Turner syndrome average more than 3 SD below the normal mean height, short stature is the major concern of patients with this disorder. The outcome variables include data on both safety and efficacy, and will allow an assessment of the risk to benefit ratio of this new treatment. The third clinical trial tests the hypothesis that supplemental GH will increase the adult height of children with non-GH-deficient short stature (constitutional, familial, or idiopathic short stature). The fourth clinical trial tests the hypothesis that recombinant human insulin-like growth factor-1 can increase the growth velocity of children with Laron short stature and of idiopathic short stature. The number of short children in the United States who are potential candidates for treatment is about 1,000,000, which underscores the need for a rigorous assessment of risk-benefit. Thus, the answers sought by these trials have important clinical, public health, and scientific significance.

New Treatment for Hypoparathyroidism. Hypoparathyroidism is one of the few remaining hormonal deficiencies for which replacement therapy with the missing hormone is not available. Moreover, the existing treatment, vitamin D or its analogues and calcium, lacks the full renal calcium-retaining action of parathyroid hormone (PTH), and thus treated patients often exhibit hypercalciuria. To test the hypothesis that treatment with PTH 1-34 can achieve simultaneous normalization of both serum and urine calcium, this group has conducted a randomized crossover trial of PTH 1-34 compared with calcitriol in patients with chronic hypoparathyroidism. The study showed that once-daily treatment with PTH 1-34 maintained both serum and urine calcium within the normal range. Biochemical markers of bone turnover increased significantly during PTH 1-34 treatment. Although rare, hypoparathyroidism represents a unique opportunity for the scientific study of the role of parathyroid hormone in human bone and calcium metabolism. The knowledge gained through such study may have implications beyond this particular disorder, such as to patients with osteoporosis or other metabolic bone disorders.

Clinical Studies in Turner Syndrome. Studies of a large cohort of girls with Turner syndrome have yielded new behavioral findings during the previous year. Analysis of behavior and social relationships in Turner girls revealed weaker social relationships, school performance, and self-esteem compared to control girls matched for age, socioeconomic status, and verbal IQ. With administration of estrogen beginning at age 12, however, we observed improved self-concept, both by self and parental report, so that by age 14-15 the girls with Turner syndrome resembled the normal controls. This improvement was not seen in a comparison group of Turner girls in whom estrogen treatment was delayed. These findings underscore the need to initiate estrogen replacement therapy by age 12-14 years in this population. Second, cognitive studies of the Turner subjects has shown a characteristic profile of impaired performance on tests of spatial recall, recognition, and reasoning despite normal verbal ability and performance. This cognitive profile was consistent from childhood through adolescence. The magnitude of the impairment was about one standard deviation unit, which implies that about 16% of Turner girls fell below the lower 95% confidence limit for normal girls. These cognitive findings suggest that spatial abilities depend on X-chromosome genes. Ongoing studies are attempting to localize these genes by determining the relationship between genotype and cognitive phenotype in Turner girls with unusual karyotypes involving partial deletion of one X-chromosome. From a social and educational standpoint, these studies suggest subtle differences in the social and cognitive performance of girls with Turner syndrome that may respond to educational approaches that are more closely tailored to their particular needs.

Development of an Ultrasensitive Estradiol Assay. Girls enter puberty and complete their growth earlier than boys. The epiphyses of girls also ossify more rapidly than those of boys, which leads to the need for separate standards for determining the bone age of girls and boys. This group hypothesized that prepubertal girls have higher levels of estradiol than prepubertal boys, and that this higher estradiol level explains the more rapid bone maturation and earlier onset of puberty. This hypothesis has not previously been testable due to the insufficient sensitivity of estradiol assays. To test this hypothesis, this group developed a recombinant cell bioassay for estradiol with a detection limit < 0.02 pg/mL, more than 100-fold more sensitive than previous assays. The assay employs a strain of yeast that has been genetically engineered for extreme sensitivity to estrogen. With this assay, the group showed that girls do indeed have significantly higher estradiol levels than boys (mean level 0.6 pg/Ml versus 0.08 pg/mL). This new assay will greatly facilitate study of the role of estradiol in normal children. Other applications include premature thelarche, prepubertal gynecomastia, childhood obesity (in which elevated estrogen may contribute to increased growth rate and bone maturity), the monitoring of treatment of precocious puberty and of estrogen-dependent neoplasms (such as breast cancer), and the menopause.

Treatment of LHRH-dependent Precocious Puberty. The efficacy of LHRH analog in the treatment of precocious puberty was first established by this group, who are completing study of a large cohort of affected children. The first 44 of these children have attained adult height or near adult height (within 2 cm). These children's final height (approximately - 1 SD compared to the normal population) significantly exceeded their pretreatment predicted height (-2 SD) but fell short of their target height based upon the height of their parents (0 SD). However, since these first patients had a delay between pubertal onset and treatment that averaged more than 3 years, it is likely that subsequent patients will come even closer to attaining their full genetic height potential.

Mutations of the LH Receptor in Familial Male Precocious Puberty. Research on early puberty has led to recent advances in both pathophysiology and treatment. To test the hypothesis of a constitutively activated LH receptor in boys with LHRH-independent familial male precocious puberty (FMPP), portions of the LH receptor gene were amplified by the polymerase chain reaction and sequenced. A mutation was observed in a region thought to be involved in signal transduction in other seven-transmembrane, g protein-coupled receptors. Expression of the mutant receptor in cultured cells caused constitutive activation of adenylate cyclase in the absence of the hormonal ligand. More recently, eight different constitutively activating mutations have been found among more than forty FMPP families, and four other mutations have been found in other laboratories. These observations establish activating mutations of g protein-coupled hormone receptors as a mechanism of human disease. Moreover, since 11 of the 12 known mutations occur in a 40-amino acid region from position 542 to 581, these observations define a domain of the LH receptor that regulates signal transduction and hence is critical for the hormonal regulation of human reproduction.

Computer analysis suggested that the activating mutations in FMPP disrupt the helical conformation in the distal third intracellular loop and sixth transmembrane domains. Although no naturally occurring mutation at Phe 576 had been observed in FMPP, the analysis suggested the possibility of activating mutations at this site. Cells transfected with the Phe 576 Ile and Phe 576 Gly mutations (generated by site-directed mutagenesis) did indeed show constitutive activation, analogous to the original FMPP mutation, Asp 578 Gly. By contrast, the Phe 576 Glu mutation, which was predicted to induce a totally helical conformation in the distal third intracellular and sixth transmembrane domains, created a null receptor that did not bind human chorionic gonadotropin (HCG) nor increase cyclic adenosine monophosphate (cAMP) nor inositol phosphate (IP) levels in response to hCG. Future studies will attempt to refine the model for mutant receptors to determine the precise structural correlates of receptor activation.

Mutations of the LH Receptor in Leydig Cell Hypoplasia. This group has also examined the hypothesis that Leydig cell hypoplasia (LCH), a disorder that results in boys with incompletely formed external genitalia, might be caused by inactivating mutations in the same LH receptor gene. To date several mutations have been identified that appear to explain this disorder -one mutation produces a truncated receptor at position 545, a second introduces a missense mutation at position 616, and a third causes deletion of exon 8, which encodes part of the extracellular LH-binding region. Each of these mutations alters both the hormone-binding and the signal-transducing ability of the LH receptor. Interestingly, 2 of these inactivating mutations are in the same general region (positions 545 and 616) as the activating mutations (positions 542 to 581) in FMPP. Future studies by this group will examine other mutations that can give rise to the same phenotypes, and will attempt to determine the precise structural requirements for ligand-binding, on the one hand, and signal transduction via G-proteins on the other. Since the 3-dimensional signal transduction domains are likely to be conserved among the large family of G protein-coupled receptors, study of this rare experiment of nature could shed light on this basic mechanism of hormone signal transduction.

Treatment of Familial Male Precocious Puberty. A new approach to the treatment of FMPP has proven effective in normalizing the rate of growth and bone maturation and in preventing acne, spontaneous erections, and aggressive behavior. The treatment combines an antiandrogen (spironolactone) with an inhibitor of androgen-to-estrogen conversion (testolactone) to block estrogen effects on epiphyseal growth. At present, the long-term effects of this new treatment are being evaluated, and attempts are being made to improve treatment with more potent aromatase inhibitors, and to treat secondary central precocious puberty, when it complicates FMPP, with an LHRH analog as well.

Treatment of Congenital Adrenal Hyperplasia. Accelerated growth in FMPP is caused by elevated secretion of androgen and estrogen, which is the same mechanism that causes accelerated growth and early epiphyseal fusion in congenital adrenal hyperplasia (CAH). Thus, these workers have begun a new trial to test whether the combination of antiandrogen (flutamide) and an inhibitor of androgen-to-estrogen conversion (testolactone) would be effective in CAH. The short-term results of this regimen have shown improved control of growth rate, weight gain, and bone maturation and reduced risk of glucocorticoid complications through reduced hydrocortisone dose. Longer term study of this new regimen is ongoing.

Laboratory studies in mice with CAH due to 21-hydroxylase deficiency are testing the hypothesis of an increased number of hypothalamic CRH neurons due to decreased glucocorticoid negative feedback in utero. This hypothesis would help explain the clinical observation of apparent resistance to hypothalamic suppression by glucocorticoid in CAH. A second initiative is attempting to correct CAH in 21-hydroxylase-deficient mice through gene therapy.

Diagnosis of Delayed Puberty. The differential diagnosis of delayed puberty continues to pose a difficult clinical problem for the endocrinologist. Currently, no definitive means exist to distinguish the child who has constitutional delay of puberty, and therefore will enter puberty spontaneously, from the child who has isolated hypogonadotropic hypogonadism, and will require hormonal treatment to induce puberty. Recent studies in the prepubertal rhesus monkey have shown that pulsatile administration of N-methyl-D-aspartate (NMDA), as well as other neuromodulators such as cholecystokinin, can trigger pulsatile release of LHRH. This suggests that it may be feasible to develop a functional test of the integrity of the LHRH neuron. The group plans to examine the feasibility of a clinical test of LHRH neuron function, based upon these observations. The hypothesis is that such a test would reveal integrity of the LHRH neuron in children with constitutional delay of puberty, and show deficient LHRH neuron responsiveness in isolated hypogonadotropic hypogonadism.

Cushing's Syndrome. The Developmental Endocrinology Branch has over the past decade made major contributions to the differential diagnosis of hypercortisolism. The CRF test and inferior petrosal sinus sampling (IPSS) have been established as major diagnostic tools in the identification of pituitary adenomas causing Cushing's disease. Recently, the dexamethasone-suppressed CRF test has been developed by Dr. Jack Yanovski as a uniquely powerful tool for distinguishing pseudo-Cushing's states from Cushing's syndrome. This test is important because it is often very difficult on clinical grounds alone to distinguish mild Cushing's syndrome from hypercortisolism in obese patients with pseudo-Cushing's states such as depression, alcoholism or poorly controlled diabetes. The hypercortisolism in these latter conditions is thought to be due to increased hypothalamic secretion of CRF in the context of an hypothalamic-pituitary-adrenal axis that is otherwise normally constituted. In contrast, hypothalamic CRF secretion is suppressed by the hypercortisolism in true Cushing's syndrome and ACTH-secreting pituitary adenomas are less responsive to the negative feedback of glucocorticoids than normal corticotrophs. The dexamethasone suppression test involves two days of low dose dexamethasone administration followed by a CRF stimulation. An elevated cortisol value measured 15 minutes after CRF has been shown to be 100% specific and sensitive for the diagnosis of Cushing's syndrome.

In additional studies, Yanovski and colleagues have investigated potential hyper-secretion of hypothalamic ACTH-releasing factors in the pathogenesis of pituitary microadenomas causing Cushing's disease. While CRF has been shown to be reduced in both cerebrospinal fluid and IPS plasma in patients with Cushing's disease, the status of vasopressin, another potent ACTH secretagogue was unknown. To test the hypothesis that vasopressin (VP) hyper-secretion is present in Cushing's disease, these investigators compared bilateral inferior petrosal sinus and peripheral VP and ACTH levels in 23 patients with Cushing's disease, 16 patients with the syndrome of ectopic ACTH, and 9 normal volunteers, before and after CRF. Unexpectedly, it was found that almost all subjects demonstrated significant inter-petrosal lateralization for both VP and ACTH secretion, with the two hormones preferentially secreted together from one side of the petrosal sinus. The physiological reason for this pattern of pituitary secretions is not known. In patients with Cushing's disease, the lateralization pattern tended to identify the tumor-containing side of the pituitary, but the correlation did not achieve statistical significance. There were no significant differences in basal or stimulated VP levels in any of the three patient groups, indicating that hypersecretion of VP is not likely to contribute to the etiology of Cushing's disease.

In current clinical studies, Dr. Lynnette Nieman is evaluating the effectiveness of jugular vein sampling as a substitute for the more invasive and technically demanding IPSS for the definitive demonstration of central versus peripheral ACTH-dependent hypercortisolism. Validation of the jugular ACTH sampling approach would allow local medical centers without expertise in IPSS to directly localize the source of ACTH hypersecretion in ACTH-dependent hypercortisolism, whereas at present the IPSS test is offered in only a few highly specialized centers. In additional new clinical studies, Drs. Chrousos and Papanicolaou have demonstrated that the cytokine interleukin-6 (IL-6) is a potent stimulator of ACTH release in normal subjects and are now testing the hypothesis that administration of IL-6 may serve as a novel provocative test of the CRF neuron allowing the differentiation of patients with true Cushing's disease - expected to demonstrate suppressed CRF neuron responses, from those with pseudo-Cushing's states - which are predicted to have normal CRF responsiveness. These investigators have also shown that glucocorticoids regulate IL-6 levels in human subjects and that IL-6 levels are reduced in patients with Cushing's syndrome and return to normal after the surgical cure of hypercortisolism. In fact, they suggest that a rebound elevation in circulating IL-6 levels immediately after surgical cure of hypercortisolism may be the cause of the systemic symptoms typical of the glucocorticoid withdrawal syndrome. Thus, IL-6 participates in an immune-endocrine feedback loop, involving CRF, ACTH and cortisol.

The Section on Pediatric Endocrinology, headed by George P. Chrousos, investigates the hypothalamic-pituitary-adrenal (HPA) axis and the reproductive system from physiological and pathophysiological perspectives. During the past year, corticotropin-releasing hormone (CRH) was used to elucidate important functional characteristics of the HPA axis under various physiological conditions, such as physical and emotional stress, and in major endocrine diseases, psychiatric disorders and autoimmune states. Also, the long sought tissue CRH was identified as an important component of the inflammatory/immune response and ovarian and endometrial functions. The molecular mechanisms of the hereditary diseases congenital isolated glucocorticoid deficiency, glucocorticoid resistance and luteinizing hormone (LH) resistance were elucidated by identifying pathogenetic defects of the ACTH, glucocorticoid and LH receptor genes, respectively.

Role of CRH in Psychiatric and Developmental Disorders. This group has used CRH productively to probe central mechanisms in patients with atypical, seasonal depression, the chronic fatigue/fibromyalgia syndromes and the postpartum blues/depression syndromes. These patients have responses to CRH indicative of mild, sustained hypocortisolism of central nervous system etiology, suggesting that a spectrum of diseases exist, in which the pathophysiology is characterized by hypofunction of the central stress system. These syndromes contrast to melancholic depression, panic anxiety and anorexia nervosa, in which hyperactivity of the CRH system were earlier demonstrated by this group. The similarities between patients with Cushing's syndrome and atypical depression, also shown by this group, probably stem from a similar pathologic decrease in CRH secretion. Recently, preadolescent and adolescent girls with a history of sexual abuse were shown by Chrousos and his colleagues to have an HPA axis response to CRH similar to that of patients with melancholic depression or recovering anorexics. These findings suggest that major stress in children may have long-standing effects on the HPA axis accompanied by effects on the psyche. Indeed, dysthymia was found in the majority of the sexually abused children studied. In a social nonhuman primate model this group demonstrated effects of parental abuse not only on the HPA axis but also on inhibiting growth. This group now studies the ability of a nonpeptide CRH antagonist, which crosses the blood barrier, to inhibit the stress-mimicking properties of CRH, in an effort to find a new class of pharmacologic agents for the treatment of diseases characterized by CRH hypersecretion, such as melancholic depression and anorexia nervosa.

Regulation of Human (h) CRH Gene Expression. In their efforts to understand the regulation of human CRH synthesis and secretion, this group cloned and sequenced the 5' regulatory region of the HCRH gene. Computer search for the presence of consensus regulatory elements revealed a cyclic AMP responsive element, 8 perfect AP1 sites, 5 perfect half palindromic estrogen responsive elements (EREs) and absence of any discernible consensus for the glucocorticoid response element. Two overlapping segments, the proximal ~0.9 kb (containing 1 pair of perfect half palindromic EREs) and the ~2.4 kb (including the former and containing an additional pair of perfect half palindromic EREs) of the 5' flanking region of the HCRH gene, conferred estrogen-mediated transcriptional enhancement to a homologous or heterologous promoter. This may have important physiological implications and may constitute the basis of sexual dimorphism in the expression of the CRH gene in the CNS and the periphery, pertaining to the existing gender differences in the stress response and immune regulation. Indeed, female rodents and humans have increased production of hypothalamic and peripheral CRH.

"Tissue" or "Immune" CRH at Sites of Inflammation. The Chrousos group demonstrated the presence of immunoreactive CRH with chromatographic mobility similar to that of synthetic CRH 1-41 in inflammatory tissues of the rat and human (neurogenic inflammation, synovial fluid in experimental and natural arthritis, autoimmune lymphocytic inflammation in Hashimoto thyroiditis, and inflamed ophthalmic tissues in experimental uveitis and inflamed intestinal tissues in ulcerative colitis). They reported that the concentrations of this "immune" CRH were quite elevated locally, and that it exerted major proinflammatory effects in vivo and in vitro. Like other inflammatory autacoids, immune CRH does not circulate in significant amounts in plasma, perhaps because it is bound by CRH-binding protein and taken up or metabolized rapidly. Receptors for CRH were identified in inflammatory sites, similar in binding characteristics and size to those of the pituitary and belonging to the central nervous system receptor type 1. Immunoneutralization of CRH resulted in marked attenuation of acute inflammation and of chronic inflammation in its early phase. Part of the proinflammatory actions of CRH were exerted by degranulation of perivascular mast cells. The Chrousos group has, thus, defined the presence, chemical identity and function of "tissue CRF", a substance that eluded active research by many groups for over 2 decades. They now study its regulation and relative importance in inflammation and inflammatory disease, employing a nonpeptide CRH receptor 1 antagonist.

Interactions Between the Immune System and the HPA Axis. Several cytokines, including interleukin 1, tumor necrosis factor-α (TNF-α), and interleukin-6, as well as several lipid mediators of inflammation, including platelet activating factor and several eicosanoids, cause profound stimulation of the HPA axis, primarily by activation of the CRH neuron. Interleukin-6 was recently studied by the Chrousos group in humans. This cytokine was extremely potent in activating the HPA axis of these patients and at high doses it caused vasopressin secretion. At all doses it was quite benign, unlike TNF-α and interleukin-1, which caused hypotension and vascular leak phenomena. Thus, interleukin-6 in man may play a major role in the communication of the immune and endocrine systems and may participate in the pathogenesis of the syndrome of inappropriate secretion of antidiuretic hormone. Also, these findings suggest that recombinant interleukin-6 could be employed in a novel provocative test of the CRH neuron, in disorders associated with potential dysfunction of this neuron. Preliminary information suggests that interleukin-6 may be able to differentiate patients with Cushing syndrome from patients with Pseudocushing's states (psychiatric hypercortisolism) and patients with melancholic from patients with atypical depression.

It appears that the CRH neuron is a link between the immune and central nervous systems, by which cytokines/lipids stimulate CRH-mediated glucocorticoid-induced negative feedback of the inflammatory/immune response. Disturbance in the balance between the immune system and the HPA axis can lead to severe disease. This group recently demonstrated that glucocorticoids in conjunction with catecholamines influence the production of interleukin-12 and interleukin-10 that causes a shift from a cellular to humoral immunity pattern rather than general immunosuppression as originally thought. Subnormal secretion of glucocorticoids and inadequate suppression of the inflammatory response by these hormones were found to be a pathogenetic factor in the propensity of animals and humans to develop severe inflammatory disease. This was tested in patients with rheumatoid arthritis and other autoimmune states. Indeed, patients with rheumatoid arthritis were shown to have subnormal HPA axis responses to concurrent inflammatory stimuli.

Local Production of CRH in the Ovary and Endometrium. The ovary is a likely site of CRH production and action, because two of its key functions, ovulation and luteolysis, are reminiscent of an aseptic inflammatory response. This group evaluated the potential role of CRH in the ovary by first examining the ovaries of adult cycling female Harlan-Sprague Dawley rats by immunohistochemistry for the presence of immunoreactive CRH (ircrh) at the various phases of the estrous cycle. They detected CRH in the cytoplasm of theca and stroma cells and in a subpopulation of cells within the corpora lutea, at all phases of the estrous cycle, and also measured high ircrh concentrations in extracts of rat ovaries by a specific radioimmunoassay. Additional studies with human ovaries, also demonstrated the presence of ircrh in theca and stroma cells. In further support of a regulatory role of CRH in the endocrine or reproductive functions of the ovary, CRH receptors were localized and characterized in the rat ovary. Auto-radiographic studies indicated that CRH binding sites were located primarily in ovarian theca and stroma and were quite prominent in areas surrounding follicles. These findings suggest that CRH may play an auto/paracrine, regulatory role, perhaps related to its proinflammatory properties, in one or more of the various inflammatory processes of the ovary, such as ovulation, follicular atresia, and/or luteolysis. It is intriguing to consider potential pathophysiologic involvement of CRH in states of ovarian dysfunction. Indeed, abnormally low secretion of CRH was demonstrated in ovaries from women with the polycystic ovarian syndrome, which the CRH receptor type found in the ovaries was the same as that found in the anterior pituitary and central nervous system.

The endometrium is also a likely site of CRH production and action, because both blastocyst implantation and menstruation are inflammatory phenomena. This group localized CRH in the glandular cells of the human endometrium throughout the menstrual cycle and in the stromal cells and local macrophages in the luteal phase. Functional studies are now in progress employing rat blastocyst implantation as a model.

Molecular Analysis of the ACTH Resistance Syndromes. ACTH regulates glucocorticoid secretion by the adrenal cortex via its recently cloned receptor. This membrane protein belongs to the superfamily of G protein-coupled receptors, forming its own distinct class with the receptors for αMSH. This group mapped the gene of the ACTH receptor in the short arm of chromosome 18 and studied families with hereditary isolated glucocorticoid deficiency (congenital insensitivity to ACTH) for potential defects of this gene. They found that point mutations resulting in premature termination of the receptor or nonconservative amino acid substitutions in key functional areas of the molecule cause the disease. Heterozygote carriers are clinically healthy but revealed by CRH testing, since they have markedly exaggerated responses of ACTH to this hormone. In a similar syndrome, which in addition to ACTH resistance, includes alacrima and achalasia (Triple A Syndrome), the Chrousos group found an intact ACTH receptor gene. However, obligate heterozygotes for this syndrome also had abnormal CRH stimulation tests.

Description and Molecular Analysis of the Aromatase Excess Syndrome. Normally, estrogen is produced from androgen precursors with the help of an enzyme, aromatase. This group recently described the third family in the world with the "aromatase excess syndrome." This family had members affected in 3 generations. The transmission was autosomal dominant. A boy with prepubertal gynecomastia and a girl with isosexual precocious puberty were the index cases in this family. Their father had also gynecomastia and short stature, while their maternal grandmother and aunt had massive macromastia that necessitated reduction mammoplasty. The affected children responded well to treatment with an aromatase inhibitor, while the molecular defect was found to be the ectopic expression of an aberrant novel promoter which we cloned and sequenced.

Molecular Analysis of the LH Resistance Syndromes. LH regulates testosterone secretion by Leydig cells and ovulation and corpus luteum formation by the ovarian follicle via its recently cloned receptor. This membrane protein belongs to the superfamily of G protein coupled receptors, forming its own distinct class with the receptors for the other glycoprotein hormones. This group studied families with hereditary primary Leydig cell failure with genetic male members that presented with phenotypically female external genitalia or micropenis and genetic females with primary amenorrhea and infertility. They found that point mutations resulting in premature termination of the receptor or nonconservative amino acid substitutions in key functional areas of the receptor molecule caused the disease. Heterozygotes were clinically healthy. Genetic females with complete absence of the LH receptor developed normally in utero and had a normal pubertal development, but remained anovulatory.

Molecular Analysis of Glucocorticoid Resistance Syndromes. Glucocorticoids act upon their target tissues by modulating gene expression. This is achieved by the interaction of a glucocorticoid-glucocorticoid receptor complex with specific sites in the nucleus (hormone-responsive elements), which regulate the expression of genes encoding function-specific proteins. These workers studied several kindreds with glucocorticoid resistance. These patients presented with hypertension and hypokalemic alkalosis and/or hyperandrogenism, without Cushing's syndrome stigmata or adrenal insufficiency. In an attempt to understand fundamental principles of glucocorticoid action and the molecular mechanisms of glucocorticoid resistance, Chrousos and colleagues obtained permanent cell lines from these patients. Dr. Chrousos' group has examined the molecular mechanisms of glucocorticoid resistance in several kindreds. Affected members of one of them had approximately half the normal glucocorticoid receptors concentration, and the proband was a young woman with hyperandrogenism. Using the polymerase chain reaction and DNA from affected members, this group amplified and sequenced each of the nine exons of the glucocorticoid receptor gene, along with their 5'- and 3'-flanking regions. They discovered a 4-base deletion at the 3'-boundary of exon 6 in one glucocorticoid receptor allele, which removed a donor splice site in all affected members. By sequencing of amplified cDNA, only the mRNA transcripts of the undeleted allele were detected in these patients, which explains the 50 percent reduction in glucocorticoid receptor concentrations, and the autosomal dominant transmission. Another interesting patient with glucocorticoid resistance was studied recently by the Chrousos group. This patient had approximately 50% reduction of his glucocorticoid receptors and was the only member affected in his family. He had a novel de novo germ-line mutation in the hinge region of the ligand binding domain of the receptor, which resulted in an inactive mutant receptor with marked dominant negative activity on the normal receptor. In his early thirties, this patient presented with hypertension and while on treatment developed pituitary Cushing's disease. His pituitary adenoma had abnormal expression of the p53 gene. Thus, generalized glucocorticoid resistance was associated with pituitary corticotropinoma formation, with the glucocorticoid resistance mutation acting as a first "hit". Interestingly, when this group examined large pituitary corticotropinomas for presence of somatic mutations of the glucocorticoid receptor, they found that one of four tumors had such a mutation causing tumor-specific glucocorticoid resistance, which could explain its unopposed growth. Thus, this group found different mutations associated with glucocorticoid resistance, causing different clinical phenotypes and associated with different modes of genetic transmission. These studies unravelled not only basic information on the functional properties of the glucocorticoid receptor, but also suggested that partial, tissue-specific glucocorticoid resistance might be involved in the pathophysiology of major human diseases, with widely varying and common manifestations, including affective disorders, such as depression, autoimmune disorders, such as steroid resistant asthma and rheumatoid arthritis, and formation of tumors from tissues whose growth is normally opposed by glucocorticoids.

A Natural Determinant of Glucocorticoid Sensitivity in Human Tissues and Its Role in Human Pathophysiology. The glucocorticoid receptor gene, located in the long arm of chromosome 5, has two protein products by alternative splicing. First, the classic glucocorticoid receptor, designated GRα and, second, a nonligand binding variant, called GRϐ. This group demonstrated that both isoforms are expressed in human tissues and that the nonligand binding receptor is present at high concentrations in normal human tissues and exerts dominant negative effects on the classic receptor. Since, alteration in the glucocorticoid receptor gene splicing would produce different ratios of GRα to GRϐ, one can envision tissues to change their sensitivity to glucocorticoids via this mechanism. This might have major implications to human pathophysiology, since both glucocorticoid resistance and glucocorticoid hypersensitivity could produce disease states. The latter could be involved in metabolic disorders, such as visceral obesity or syndrome X, affective disorders, such as depression, and immune disorders characterized by suppression of the inflammatory/immune response and vulnerability to infectious diseases and/or tumors. This group showed that glucocorticoid resistant asthma type II was associated with a major switch of the GRα to GRβ ratio towards GRβ predominance, explaining the profound resistance of these patients' cells to glucocorticoids. A similar switch was found in cells from glucocorticoid resistant New World monkeys. Recently, this group demonstrated that one of the proteins of the HIV-1 called vpr altered the sensitivity of human cells to glucocorticoids in a way favoring viral replication and causing acquired peripheral glucocorticoid resistance, a phenomenon reported in AIDS patients.

Chromosomal Mapping of the Carney Complex Locus. This group studied the genetics of Carney complex, a multiple neoplasia and lentiginosis syndrome involving the adrenal glands and transmitted in an autosomal dominant fashion. They mapped the defective gene within a 4cMorgan region of the short arm of chromosome 2 in band p16. They also performed cytogenetic studies on the tumors of Carney patients and demonstrated that the gene was most likely an oncogene rather than a tumor suppressor gene. At this time, this group examines candidate genes in the area and identifies the appropriate yeast artificial chromosomes that would lead to isolation of the gene.

The Section of Endocrine Physiology, directed by Dr. Greti Aguilera, has two major research interests, a) the neuroendocrine mechanisms of the stress response with emphasis in the regulation of the hypothalamic pituitary adrenal (HPA) axis, and b) the role of the renin-angiotensin system in the regulation of mineralocorticoid secretion and the effects of angiotensin II (Ang II) in other endocrine and non-endocrine systems. This group pioneered studies on the role of type 2 Ang II receptors during fetal development. Research by this group showed that the temporal pattern of expression of these receptors during postnatal development follows closely the differentiation of neural pathways in the brain, and nephron differentiation in the kidney suggesting a role for Ang II in development. In the field of mineralocorticoid secretion, two important recent findings have been the demonstration of developmental changes in the regulation of aldosterone secretion, and of marked inhibitory effects of chronic stress on the expression of the rate limiting enzyme in aldosterone biosynthesis, aldosterone synthetase. Investigation of the molecular mechanisms of these effects will be the subject of future studies. Current research also focus on elucidating the physiological role of central Ang II receptors. Studies during the past year showed that blockade of Ang receptors type-1 (AT) by icv injection of specific antagonist, Losartan, markedly reduces the increases in plasma catecholamines in response to stress, while having no effect on the HPA axis responses. Current and future research in this area includes blockade and overexpression of AT1 receptors directly in the hypothalamic paraventricular nucleus using gene transfer techniques.

In the area of the **mechanisms of adaptation to stress**, studies of this group have been responsible for the characterization of the responses of different components of the HPA axis during different types of stress. This group has demonstrated that vasopressin plays a predominant role in the regulation of pituitary ACTH secretion during chronic stress. This is reflected in increased VP:CRH ratios in the hypothalamus, and by a good correlation between pituitary ACTH responsiveness and the content of vasopressin receptors in the pituitary. During the last year major progress was achieved in the understanding of the regulation of pituitary CRH receptors in vivo. These studies have shown that chronic stress induces increases in pituitary CRH receptor MRNA despite sustained decreases in CRH binding. The mechanisms of these changes involves the interaction of hypothalamic CRH and VP and circulating glucocorticoids in the pituitary corticotroph. Current work is directed to investigate the molecular mechanisms of CRH receptor regulation using pituitary corticotroph cell lines and primary pituitary cell cultures. This work contributes to the current knowledge of the regulation of the HPA axis during chronic stress, and provides strong basis for future studies on the physiological role of neuropeptides in the regulation of PVN function and the molecular mechanisms of CRH and VP receptor regulation.

1996 Annual Report Bibliography for DEB, NICHD

Accili D. Insulin receptor knock-out mice. Trends Endocrinol. Metab. in press.

Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI and Westphal H. Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. Nat Genet 1996;12:106-9

Adesanya OO, Zhou J, Bondy CA. Sex steroid regulation of insulin-like growth factor system gene expression and proliferation in primate myometrium. J Clin Endocrinol Metab 1996;81:1967-74.

Adesanya OO, Zhou J, Bondy CA. Cellular localization and sex steroid regulation of insulin-like growth factor binding protein (IGFBP) mRNAs in the primate myometrium. J Clin Endocrinol Metab 1996;81:2495-2501.

Aguilera G and Kiss A. Regulation of the hypothalamic pituitary adrenal axis and vasopressin secretion: role of angiotensis II. In: Recent advances in cellular and molecular aspects of angiotensin receptors. Raizada MK, Phillips MI, Sumners C, eds. Plenum Press, New York, 1996;105-12.

Aguilera G, Kiss A, Luo X. Increased expression of type-1 angiotensin II receptors in the hypothalamic paraventricular nucleus following stress and glucocorticoid administration. J Neuroendocrinol 1995;7:775-83.

Aguilera G, Kiss A, Luo X and Sunar-Akbasak B. The renin-angiotensin system and the stress response. Ann NY Acad Sci 1995;771:173-86.

Aguilera G, Kiss A and Sunar-Akbasak B. Hyperreninemic hypoaldosteronism following chronic stress in the rat. J Clin Invest 1995;96:1512-9.
Aguilera G, Kiss A, Lu A, Camacho C. Regulation of adrenal steroidogenesis during chronic stress. Endocrine J, in press.

Aguilera G, Rabadan-Diehl C, Luo X and Kiss A. Regulation of pituitary ACTH secretion during stress: role of corticotropin releasing hormone and vasopressin receptors. In: Stress: molecular genetic and neurobiological advances. Gordon and Breach, New York, in press.

Arai K, Tsigos C, Suzuki Y, Listwak S, Zachman K, Zangeneh F, Rapaport R, Chanoine JP, Chrousos GP. No apparent mineralocorticoid receptor defect in a series of sporadic cases of pseudohypoaldosteronism. J Clin Endocrinol Metab 1995;80:814-7.

Avgerinos PC, Nieman LK, Oldfield EH, Cutler GB Jr. A comparison of the overnight and the standard metyrapone test for the differential diagnosis of Cushing syndrome. Clin Endocrinol, in press.

Avila NA, Premkumar A, Shawker TH, Jones JV, Laue L, Cutler GB Jr. Testicular adrenal rest tissue in congenital adrenal hyperplasia: gray scale and color Doppler ultrasound. Radiology 1996;198:99-104.

Baker J, Hardy MP, Zhou J, Bondy CA, Lupu F, Bellve A, Efstratiadis A. Effects of an IGF-I null mutation on mouse reproduction. Mol Endocrinol 1996;10:903-18.

Bamberger CM, Bamberger AM, Chrousos GP. Dominant negative inhibitors of the glucocorticoid receptor. In: De Bellis A, Schipani E, eds. Frontiers in Endocrinology, Vol. 14. Serono Symposia Publication, Rome, Italy, 1995;129-39.

Bamberger CM, Bamberger AM, Wald M, Chrousos GP, Schulte HM. Inhibition of mineralocorticoid activity by the β-isoform of the human glucocorticoid receptor. J Steroid Biochem Mol Biol, in press.

Bamberger CM, Chrousos GP. The glucocorticoid receptor and RU 486 in man. Ann NY Acad Sci 1995;761:296-311.

Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity. Endo Rev 1996;17:221-44.

Barnes KM, Dickstein B, Cutler GB Jr, Fojo AT, Bates SE. The accumulation of steroids in multidrug-resistant cells and steroid antagonism of the P-glycoprotein drug efflux pump can be decreased by the PKC inhibitor, calphostin C. Biochemistry 1996;35:4820-7.

Baron J. Longitudinal bone growth: experimental methods. In: Thomas JA, ed. Endocrine Methods. Academic Press Inc, San Diego, 1996;81-8.

Baron J, Winer KK, Yanovski JA, Cunningham AW, Laue L, Zimmerman D, Cutler GB Jr. Mutations of the Ca^{2+}-sensing receptor gene cause autosomal dominant and sporadic hypoparathyroidism. Hum Mol Genet 1996;5:601-6.

Bondy CA and Zhou J. Functional correlates of IGF system expression in the murine and primate ovary. In: LeRoith D, ed. The role of IGFs in ovarian physiology. Rome: Ares Serono Publications, 1996.

Burgess ES, Dorn LD, Haaga D, Chrousos GP. Sociotropy, autonomy, stress and depression in cushing syndrome. J Nerv Ment Dis, in press.

Bruening J, Winnay J, Bonner-Weir S, Accili D and Kahn CR. Creating polygenic models of insulin resistance and NIDDM. J. Invest. Med. 1996;44(3):258A

Carter CS, DeVries AC, Taymans SE, Roberts RL, Williams JR, Chrousos GP. Adrenal corticoid hormones and the development and expression of mammalian monogamy. Ann NY Acad Sci 1995;771:82-91.

Chan WY, Cutler GB Jr. Molecular aspects of precocious puberty. Adv Mol Cell Endocrinol, in press.

Chrousos GP. Cytokine therapy in septic shock. (Letter to the Editor). N Engl J Med 1995;333:942-3.

Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med 1995;332:1351-62.

Chrousos GP, Castro M, Leung DYM, Webster E, Kino T, Bamberger CM, Elliot S, Stratakis C, Karl M. Molecular mechanisms of glucocorticoid resistance/hypersensitivity. Am J Resp Crit Care Med, in press.

Chrousos GP, McCarty R, Cizza G, Pacak K, Sternberg E, Gold PW, Kvetananski R. (eds.) Stress: mechanisms and clinical implications. Ann NY Acad Sci 1995;771: New York.

Cizza G, Nieman LK, Doppman JL, Passaro MD, Czerwiec FS, Chrousos GP, Cutler GB Jr. Factitious cushing syndrome. J Clin Endocrinol Metab, in press.

Cizza G, Gold PW, Chrousos GP. Aging is associated in the 344/N fischer rat with decreased stress responsivity of central and peripheral catecholaminergic systems and impairment of the hypothalamic-pituitary-adrenal axis. Ann NY Acad Sci 1995;771:491-511.

Crofford LJ, Sano H, Karalis K, Webster EA, Friedman TC, Chrousos GP, Wilder RL Local expression of corticotropin-releasing hormone in inflammatory arthritis. Ann NY Acad Sci 1995;771:459-71.

Cutler GB Jr. Ambiguous genitalia. In: Hurst JW ed. Medicine for the practicing physician, 4th ed. Stamford, Connecticut: Appleton Lange, 1996;617-23.

Cutler GB Jr. Central puberty in disorders with accelerated peripheral maturation. In: Plant, T.M. and Lee, P.A., eds. The neurobiology of puberty. Bristol, J Endocrinol Ltd 1995;209-15.

Cutler GB Jr. Precocious puberty. In: Hurst, J.E., ed. Medicine for the Practicing Physician, 4th ed. Stamford, Connecticut: Appleton Lange 1996;623-8.

Cutler GB Jr. Treatment of congenital adrenal hyperplasia: the case for new medical approaches. J Clin Endocrinol Metab, in press.

Czerwiec FS, Cutler GB Jr. Modern approach to corticosteroid replacement. Curr Opinion Endocrinol Diabetes 1996;3:239-46.

Di Cola G, Cool MH and Accili D. Normal metabolic responses to IGF-1 in mice lacking insulin receptors. Annual Meeting of the Endocrine Society 2:749: abstract OR66-1

Dorn LD, Burgess ES, Susman EJ, Von Eye A, DeBellis MD, Gold PW, Chrousos GP. Response to ovine corticotropin releasing hormone in depressed and nondepressed adolescents: does gender make a difference? J Am Acad Child Adolesc Psychiatry, in press.

Elenkov IJ, Papanicolaou DA, Wilder RL, Chrousos GP. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. Proc Assn Am Phys

Friedman TC, Mastorakos G, Newman TD, Mullen NM, Horton EG, Costello R, Papadopoulos NM, Chrousos GP. Carbohydrate and lipid metabolism in endogenous hypercortisolism: shared features with metabolic syndrome X and noninsulin-dependent diabetes mellitus. Endocrine Journal (Japan).

Friedman TC, Nieman LK, Yanovski JA, Doppman JL, Cutler GB Jr., Chrousos GP, Kalogeras KT. Inferior petrosal sinus arginine vasopressin concentrations in normal volunteers and patients with Cushing disease. J Clin Endocrinol Metab 1996;81:3068-72.

Gold PW, Licinio J, Wong ML, Chrousos GP. Corticotropin releasing hormone in the pathophysiology of melancholic and atypical depression and in the mechanism of action of antidepressant drugs. Ann NY Acad Sci 1995;771:716-29.

Heinrichs C, Munson PJ, Counts DR, Baron J. Patterns of human growth. Science 1995;268:442-4.

Hirschfeld S, Laue L, Cutler GB Jr., Pizzo PA. Thyroid abnormalities in children infected with human immunodeficiency virus. J Pediatr 1996;128:704.

Jezova D, Kiss A, Aguilera G. Studies on the mechanisms of the atypical plasma vasopressin responses to stress in chronically adrenal demedullated rats. Stress, in press.

Johnson EO, Decker L, Gold PW, Chrousos GP. Distribution of hippocampal mineralocorticoid and glucocorticoid receptor mRNA in a glucocorticoid resistant nonhuman primate. Steroids 1996;61:69-73.

Johnson EO, Kamilaris TC, Calogero AE, Gold PW, Chrousos GP. Effects of early parenting on growth and development in a small primate. Ped Res 1996;39:999-1005.

Johnson EO, Kamilaris TC, Carter CS, Calogero AE, Gold PW, Chrousos GP. The biobehavioral consequences of psychogenic stress in a small, social primate (*callithrix jacchus jacchus*). Biol Psychiatry

Johnson MC, Devotto LRetamales I, Kohen P, Troncoso JL, Aguilera G. IGF-I and IGF Ireceptor expression and stimulation of estradiol secretion in human corpus luteum. Fertility and Sterility 1996;65:489-94.

Kalogeras KT, Nieman LK, Friedman TC, Dopppman JL, Cutler GB Jr., Chrousos GP, Wilder RL, Gold PW, Yanovski JA. Inferior petrosal sinus sampling in normal human subjects reveals a unilateral corticotropin-releasing hormone-induced arginine vasopressin release associated with ipsilateral adrenal corticotropin secretion. J Clin Invest 1996;97:2045-50.

Karl M, Von Wichert G, Kempter E, Katz DA, Reincke M, Monig H, Ali IU, Stratakis CA, Oldfield EH, Chrousos GP, Schulte HM. Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab 1996;81:124-9.

Karl M, Saviolakis GA, Gravanis A, Chrousos GP, Margioris AN. The PC 12 rat pheochromocytoma cell line expresses the prodynorphin gene and secretes the 8 kdalton dynorphin product. Regulatory Peptides 1996;61:99-104.

Karl M, Lamberts SWJ, Koper JW, Katz DA, Huizenga NE, Kino T, Haddad BR, Hughes MR, Chrousos GP. Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assn Amer Phys, in press.

Karl M, Chrousos GP. Experimental and clinical models of CRH-induced pituitary tumors. In: Oncogenesis and molecular biology of pituitary tumors. Melmed S, ed. Front Horm Res Karger, Basel, 1996;20:54-71.

Kiss A, Palkovits M, Aguilera G. Extrahypothalamic regulation of corticotropin releasing hormone (CRH) and CRH receptor mRNA in the hypothalamic paraventricular nucleus in the rat. J Neuroendocrinol 1996;8:103-12.

Klein KO, Cutler GB Jr. Puberty. In: Hillier SG, Kitchener H, Nielson JP, eds. Scientific essentials of reproductive medicine. London: W.B. Saunders, 1996;196-207.

Klein KO, Cutler GB Jr. The hypothalamus and neuroendocrine disorders. In: Berg BO, ed. Principles of child neurology. New York: McGraw-Hill, 1996;437-57.

Klein KO, Demers LM, Santner SJ, Baron J, Cutler GB Jr, Santen RJ. Use of ultrasensitive recombinant cell bioassay to measure estrogen levels in women with breast cancer receiving the aromatase inhibitor, letrozole, J Clin Endocrinol Metab 1995;80:2658-60.

Latronico AC, Anasti J, Arnhold IJP, Rapaport R, Mendonca BB, Bloise W, de Castro M, Tsigos G, Chrousos GP. Testicular and ovarian resistance to luteinizing hormone caused by homozygous inactivating mutations of the luteinizing hormone receptor gene. N Engl J Med 1996;334:507-12.

Laue L, Cutler GB Jr. The adrenal cortex. In: Bittar EE, Bittar N, eds. Principles of medical biology. Greenwich: JA1 Press, in press.

Laue L, Merke DP, Jones JV, Barnes KM, Hill SV, Cutler GB Jr. A preliminary study of flutamide, testolactone, and reduced hydrocortisone dose in the treatment of congenital adrenal hyperplasia. J Clin Endocrinol Metab, in press.

Laue L, Wu SM, Kudo M, Bourdony CJ, Cutler GB Jr., Hsueh AJW, Chan WY. Compound heterozygous mutations of the luteinizing hormone receptor gene in Leydig cell hypoplasia. Mol Endocrinol 1996;10:987-97.

Leong G, Marton M, Wang K, Blanco JCG, Wang IM, Rolfes R, Ozato K, Segars JH. In vivo interaction between RXR and TFIIB requires 9-cis retinoic acid and the AF-2 region of RXR. Mol Cell Biol, in press???

Leong GM, Mercado-Asis LB, Reynolds JC, Oldfield EH, Chrousos GP. The effect of cushing disease on bone mineral density, body composition, growth and puberty: a report of an identical adolescent twin pair. J Clin Endocrinol Metab 1996;81:1905-11.

Leschek EW, Doppman JL, Pass HI, Cutler GB Jr. Clinical Case Seminar: Localization by venous sampling of occult chorionic gonadotropin-secreting tumor in a boy with mosaic Klinefelter syndrome and precocious puberty. J Clin Endocrinol Metab, in press.

Luo X, Kiss A, Rabadan-Diehl C, Aguilera G. Regulation of hypothalamic and pituitary corticotropin releasing hormone receptor mRNA by adrenalectomy and glucocorticoids. Endocrinology 1995;136: 3877-83.

Magiakou MA, Chrousos GP. Glucocorticoid therapy and withdrawal. IN: RC Bone. Current Practice of Medicine. Current Medicine, Inc., Philadelphia, IV 1996;6.1-6.

Magiakou MA, Mastorakos G, Rabin D, Dubbert B, Gold PW, Chrousos GP. Hypothalamic corticotropin releasing hormone suppression during the postpartum period: implications for the increase of psychiatric manifestations during this time. J Clin Endocrinol Metab 1996;81:1912-7.

Magiakou MA, Mastorakos G, Rabin D, Margioris AN, Dubbert B, Calogero AE, Tsigos C, Munson PJ, Chrousos GP. The maternal hypothalamic-pituitary-adrenal axis in third trimester human pregnancy. Clin Endocrinol 1996;44:419-28.

Makara GB, Kiss A, Lolait SJ, Aguilera G. Hypothalamic pituitary corticotroph function following shunting of magnocellular vasopressin to the hypophyseal portal circulation. Endocrinology 1996;137:580-6.

Marini JC, Bordenick S, Chrousos GP. Endocrine aspects of growth deficiency in OI. Connective tissue research 1995;31:S55-7.

Mase A, Chrousos GP. The Hypothalamic-pituitary-adrenal-glucocorticoid axis in rhematoid arthritis. J Rheumatol 1996;23:577-81.

Mastorakos G, Bouzas E, Caspi R, Friedman T, Chrousos GP. Immune corticotropin releasing hormone (CRH) is present in the eyes of and promotes experimental autoimmune uveoretinitis (EAU) in rodents. Endocrinology 1995;136:4650-8.

Mastorakos G, Chrousos GP. Chapter 79, Adrenal hyperandrogenism. In: Reproductive endocrinology, surgery, and technology. Adashi E, Rock J, Rosenwaks Z, eds. Raven Press, New York, 1995;1539-1553.

Mastorakos G, Magiakou MA, Chrousos GP. Effects of the immune/inflammatory reaction on the hypothalamic-pituitary-adrenal axis. Ann NY Acad Sci 1995;771:438-48.

Mastorakos G, Patchev VK, Chader GJ, Chrousos GP, Gaudet SJ. Differences in arylakylamine N-acetyltransferase activity between inflammatory disease-susceptible lewis and -resistant fischer rats. Neuroendocrinology 1995;62:71-8.

Mastorakos G, Scopa CD, Kao LC, Vryonidou A, Friedman TC, Kattis D, Phenekos C, Rabin D, Chrousos GP. Presence of immunoreactive corticotropin releasing-hormone (CRH) in human endometrium. J Clin Endocrinol Metab 1996;81:1046-50.

Moy E, Kimzey LM, Nelson LM, Blithe DL. Glycoprotein hormone α-subunit functions synergistically with progesterone to stimulate differentiation of cultured human endometrial stromal cells to decidualized cells: a novel role for free α-subunit in reproduction. Endocrinology 1996;137,1332-9.

Nair S, Caspi RR, Nelson LM. Susceptibility to murine experimental autoimmune oophoritis is associated with genes outside the major histocompatibility complex. Am J Reprod Immunol, in press.

Nair S, Mastorakos G, Raj S, Nelson LM. Murine experimental autoimmune oophoritis develops independently of gonadotropin stimulation and is primarily localized in the stroma and theca. Am J Reprod Immunol 1995;34:132-9.

Nelson LM, Anasti JN, Flack MR. Premature ovarian failure. In: Reproductive endocrinology, surgery, and technology. Adashi EY, Rock JA, Rosenwaks Z (eds). New York, Raven Press 1995;1393-410.

Nieman LK. Cushing's Syndrome Treatment. Current Therapy of Endocrinology and Metabolism, C. Wayne Bardin MD, Ed., 1997 edition, in press.

Nieman LK. Treatment of Cushing's Syndrome. Conn's Current Therapy, 1997 edition, in press.

Nieman LK, Cutler GB Jr. Cushing's syndrome. In: DeGroot LJ, ed. Endocrinology, 3rd ed. Philadelphia: W.B. Saunders, 1995;1741-69.

Nunez SB, Blye RP, Thomas PM, Reel JR, Barnes KM, Malley JD, Cutler GB Jr. Recovery of reproductive function in male and female rats treated with the potent aromatase inhibitor fadrozole hydrochloride. Reprod Toxicol, in press.

Nunez SB, Medin JA, Braissant O, Wahli W, Ozato K, Segars JH. The retinoid X receptor and perosisome proliferator-activated receptor activate estrogen responsive genes independent of the estrogen receptor. Mol Cell Endocrinol, in press.

Pacak K, McCarty R, Palkovitz M, Cizza G, Kopin IJ, Goldstein D, Chrousos GP. Decreased central and peripheral catecholaminergic activation in obese zucker rats. Endocrinology 1995;136:4360-7.

Papanicolaou DA, Petrides JS, Tsigos C, Bina S, Kalogeras KT, Wilder R, Gold PW, Deuster PA, Chrousos GP. Exercise stimulates interleukin-6 secretion: inhibition by glucocorticoids and correlation with catecholamines. Am J Physiol

Papanicolaou DA, Tsigos C, Oldfield EH, Chrousos GP. Acute glucocorticoid deficiency is associated with plasma elevation of interleukin-6: does the latter participate in the symptomatology of the steroid withdrawal syndrome and adrenal insufficiency? J Clin Endocrinol Metab 1996;81:2303-6.

Peeke PM, Chrousos GP. Hypercortisolism and obesity. Ann NY Acad Sci 1995;771:665-76.

Premkumar A, Stratakis CA, Shawker TH, Papanicolaou DA, Chrousos GP. Testicular ultrasound in carney complex: report of three cases. J Clin Ultrasound

Rabadan-Diehl C, Kiss A, Camacho C, Aguilera G. Regulation of messenger ribonucleic acid for corticotropin releasing hormone receptors in the pituitary during stress. Endocrinology, in press.

Rabadan-Diehl C, Lolait SJ, Aguilera G. Regulation of vasopressin V1b receptor mRNA during stress in the rat. J Neuroendocrinol 1995;7:9903-10.
Reincke M, Lehmann R, Karl M, Magiakou MA, Chrousos GP, Allolio B. Severe illness. Neuroendocrinology. Ann NY Acad Sci 1995;771:556-69.

Raj S, Nair S, Mastorakos G, Nelson LM: Anamnestic development of lymphocytic infiltration in murine experimental autoimmune oophoritis is primarily localized in the stroma and theca. Am J Reprod Immunol 1995;34:125-31.

Reinhardt RR and Bondy CA. Differential cellular pattern of gene expression for two distinct cGMP-inhibited cyclic nucleotide phosphodiesterases in developing and mature rat brain. Neuroscience 1996;72:567-78.

Rose SR, Municchi G, Barnes KM, Cassorla FG, Cutler GB Jr. Overnight growth hormone concentrations are usually normal in pubertal children with idiopathic short stature - a clinical research center study. J Clin Endocrinol Metab 1996;81:1063-8.

Ross JL, McCauley E, Roeltgen, Long L, Kushner H, Feuillan P, Cutler GB Jr. Self-concept and behavior in adolescent girls with Turner syndrome: potential estrogen effects. J Clin Endocrinol Metab 1996;81:926-31.

Ross JL, Roeltgen D, Cutler GB Jr. The neurodevelopmental transition between childhood and adolescence in girls with Turner syndrome. In: Albertsson-Wikland, K. ed. Turner Syndrome in a life span perspective: research and clinical aspects. Amsterdam: Elsevier Press, 1995;297-308.

Sartor O, Cutler GB Jr. Treatment of Cushing's syndrome with the antiglucocorticoid mifepristone (RU486). Clin Obstet Gynecol 1996;39:506-10.

Schoen TJ, Bondy CA, Zhou J, Dhawan R, Mazuruk K, Arnold DR, Rodriguez I, Waldbillig RJ, Beebe DC, Chader GJ. Differential expression and in situ localization of insulin-like growth factor binding protein-2 in developing chick ocular tissues. Invest Ophthalmol Vis Sci 1995;36:2652-62.

Segars JH. Endometriosis and nuclear hormone receptors. In: Diamond M, Osteen K, eds. Endometrium and Endometriosis. Cambridge: Blackwell Science, in press.

Sharara FI & Nieman LK. Growth hormone receptor mRNA expression in leiomyoma and surrounding myometrium. Am J Obstet Gynecol 1995;172:814-9.

Stratakis CA, Carney A, Ping J, Papanicolaou DA, Karl M, Kastner DL, Pras E, Chrousos GP. Carney complex, a familial multiple neoplasia and lentiginosis syndrome: analysis of 11 kindreds and linkage to the short arm of chromosome 2. J Clin Invest 1996;97:699-705.

Stratakis CA, Chrousos GP. Neuroendocrinology and pathophysiology of the stress system. Ann NY Acad Sci 1995;771:1-18.

Stratakis CA, Jenkins RB, Pras E, Mitsiadis CS, Raff SB, Stalboerger PG, Tsigos C, Carney A, Chrousos GP. Cytogenetic and mircosatellite alterations in tumors from patients with the syndrome of myxomas, spotty skin pigmentation, and endocrine overactivity (carney complex). J Clin Endocrinol Metab, in press.

Stratakis CA, Mitsiades NS, Chrousos GP, Margioris AN. Dopamine affects rat placenta opioids in a receptor- and opioid type-specific manner. Eur J Pharmacol, in press.

Stratakis CA, Mitsiadis NS, Sun D, Chrousos GP, O'Connell A. Recurring oral giant cell lesion in X-linked hypophosphatemic rickets: clinical manifestation of occult hyperparathyroidism? J Peds 1995;127:444-6.

Stratakis CA, Vamvakopoulos NC, Chrousos GP. Corticotropin-releasing hormone, stress and depression. In: Bailly D, ed. Neuroendocrine aspects of mood disorders. Ardix médical, Levallois 1995;119-52 (in french).

Taymans SE, DeVries AC, DeVries MB, Nelson RJ, Friedman TC, de Castro M, Detera-Wadleigh S, Carter CS, Chrousos GP. The prairie vole (*microtus ochrogaster*) as a model of glucocorticoid resistance. Gen Comp Endocrinol

Theoharides TC, Spanos C, Pang Z, Alferes L, Ligris K, Letourneau R, Rozniecki JJ, Webster E, Chrousos GP. Stress-induced intracranial mast cell degranulation. A corticotropin-releasing hormone-mediated effect. Endocrinology 1995;136:5745-50.

Tsigos C, Chrousos GP. Stress, endocrine manifestations and diseases. Chapter 3. In: Handbook of stress medicine. Cooper CL, ed. Boca Raton, FL: CRC Press Inc., 1995;61-65.

Tsigos C, Arai K, Latronico AC, Webster E, Chrousos GP. Receptors for melanocortin peptides in the hypothalamic-pituitary-adrenal axis and skin. Ann NY Acad Sci 1995;771:352-63.

Tsigos C, Chrousos GP. The neuroendocrinology of the stress response. Chapter 6. In: NIDA alcohol research monograph: stress, gender, and alcohol seeking behavior. Hunt WA, Zachari S, eds. CSR, Inc., Washington, DC, 1995;29:103-24.

Tsigos C, Kamilaris T, Chrousos GP. Adrenal diseases. In: Moore WT, Eastman R, eds. Diagnostic endocrinology, 2nd edition. B.C. Decker Inc., Toronto, 1996;123-156.

Tsigos C, Chrousos GP. Differential diagnosis and management of cushing's syndrome. Ann Rev Med 1996;47:443-61.

White PC, Pescovitz OH, Cutler GB Jr. Synthesis and metabolism of corticosteroids. In: Becker KL, ed. Principles and practice of endocrinology and metabolism, 2nd ed. New York: J.B. Lippincott, in press.

Winer KK, Yanovski JA, Cutler GB Jr. A short-term randomized, crossover trial of human parathyroid hormone 1-34 versus calcitriol in the treatment of hypoparathyroidism. J Am Med Assoc, in press.

Wong M, Ramayya MS, Chrousos GP, Driggers PH, Parker KL. Cloning and sequencing analysis of the human gene encoding steroidogenic factor 1. J Mol Endocrinol, in press.

Yano J, Kohn LD, Saji M, Kataoka N, Okuno A, Cutler GB Jr. A case of male-limited precocious puberty

caused by a point mutation in the second transmembrane domain of the luteinizing hormone/choriogonadotropin receptor gene. Biochem Biophys Res Commun 1996;220:1036-42.

Yanovski JA, Cutler GB Jr. The reproductive axis: pubertal activation. In: Adashi EY, Rock JA, and Rosenwaks, Z, eds.: Reproductive endocrinology, surgery, and technology. New York, Raven Press, 1996;196-207.

Yanovski JA, Friedman TC, Nieman LK, Doppman JL, Cutler GB Jr, Chrousos GP, Kalogeras KT. Inferior petrosal sinus arginine vasopressin in patients with Cushing syndrome. Clin Endocrinol, in press.

Yanovski JA, Yanovski SZ, Cutler GB, Chrousos GP, Filmer K. Differences in the hypothalamic-pituitary-adrenal axis of African American and Caucasian girls. J Pediatrics, 1996;129:130-6.

Yanovski JA, Yanovski SZ, Filmer K, Hubbard VS, Avila N, Lewis B, Reynolds J, Flood M. Differences in body composition of African American and Caucasian prepubertal girls. Am J Clin Nutr, in press.

Yanovski JA, Yanovski SZ, Friedman TC, Loh YP, Jayasvasti V, Cutler GB Jr, Chrousos GP. Etiology of the differences in corticotropin-releasing hormone-induced ACTH secretion of black and white women. J Clin Endocrinol Metab, in press.

Yanovski JA, Yanovski SZ, Harrington L, Gold PW, Chrousos GP. Differences in the hypothalamic-pituitary-adrenal axis of black and white men. Hor Res 1995;44:208-12.

Zhou J, Adesanya OO, Vatzias G, Hammond JM, Bondy CA. Selective IGF system expression during porcine ovary follicular selection. Endocrinology, in press.

DEVELOPMENTAL ENDOCRINOLOGY BRANCH

Chief, *Carolyn A. Bondy, M.D.

Senior Staff

*Greti Aguilera, M.D.
*George P. Chrousos, M.D.
*Gordon B. Cutler, M.D.
*Lynnette K. Nieman, M.D.

Scientist Emeritus

Roy Hertz, M.D., Ph.D.

Tenure-Track Investigators

Domenico Accili, M.D.

Senior Fellows

Jeffrey Baron, M.D.
Alexander Kiss, Ph.D.
Lawrence Nelson, M.D.
James Segars, M.D.

Post-Doctoral Fellows

Veronica Abad, M.D.
Oluyemisi O. Adesanya, M.D.
Giovanni Cizza, M.D., Ph.D.
Michael Collins, M.D.
Frank Czerwiec, M.D., Ph.D.
Francesco De Luca, M.D.
Paul Driggers, Ph.D.
Sharon Elliott, Ph.D.
Penelope Feuillan, M.D.
Robert Kimmel, M.D.
Gary Leong, M.D.
Ellen Leschek, M.D.
Xun Luo, M.D., Ph.D.
Rita Maity, M.D.
Deborah Merke, M.D.
Edmond Moy, M.D.
Martin Nemansky, Ph.D.
Susan B. Nunez, M.D.
Maureen Passaro, M.D.
Dimitris Papanicolaou, M.D.

Cristina Rabadan-Diehl, Ph.D.
Meera Ramayya, M.D.
Rickey Reinhardt, M.D., Ph.D.
Kristina Rother, M.D.
Domenica Rubino, M.D.
Constantine Stratakis, M.D.
Zhi-Bin Tong, M.D.
David Torpy, M.D.
Enmei Wang, M.D.
Elizabeth Webster, Ph.D.
Karen Winer, M.D.
Koichi Yano, M.D.
Jian Zhou, M.D., Ph.D.

Guest Researchers

Val Abbassi, M.D.
Peter Avgerinos, M.D.
James N. Anasti, M.D.
Fernando Cassorla, M.D.
Frank Chang, M.D.
Margaret De Castro, M.D., Ph.D.
Helen Dichek, M.D.
Lorah D. Dorn, Ph.D.
Michael Karl, M.D.
Thomas Kim, M.D.
John Marlow, M.D.
Bradley Miller, M.D.
Gilbert Mottala, M.D.
Sharmila Nair, M.D.
Judy Novosad, M.D.
Karen Oerter, M.D.
Beatriz R. Olson, M.D.
Pamela Peeke, M.D.
Owen Rennert, M.D.
Edwin Robins, M.D.
Susan Rose, M.D.
Judith L. Ross, M.D.
Robert J. Stillman, M.D.
Donna Vogel, M.D.
Zhi-Bin Tong, M.D.
Jack A. Yanovski, M.D., Ph.D.

Project Numbers - Annual Report 1996

DEVELOPMENTAL ENDOCRINOLOGY BRANCH (DEB)

ZO1 HD 00610-16	Growth, Puberty, Their Disorders: Physiology, Pathophysiology, and Molecular Biology Gordon B. Cutler, Jr., M.D.
ZO1 HD 00615-16	Endocrine-Immune Interactions George P. Chrousos, M.D.
ZO1 HD 00618-15	Physiology and Pathophysiology of the Hypothalamic-Pituitary-Adrenal and Gonadal Axes George P. Chrousos, M.D.
ZO1 HD 00623-13	Adrenal Physiology, Pathophysiology, and Molecular Biology Gordon B. Cutler, Jr., M.D.
ZO1 HD 00627-07	Glycoprotein Hormones: Oligosaccharide Structure and Function Diana Blithe, Ph.D.
Z01 HD 00628-07	Biological Roles and Mechanisms of Action of Insulin-Like Growth Factors (IGFs) Carolyn Bondy, M.D.
Z01 HD 00631-07	Neuroendocrine Control of the Stress Reponse Greti Aguilera, M.D.
Z01 HD 00632-07	Physiological Actions of the Renin-Angiotension-System Greti Aguilera, M.D.
Z01 HD 00633-06	Ovarian Folliculogenesis Lawrence Nelson, M.D.
Z01 HD 00636-04	Endocrinology of Reproduction in Women James Segars, M.D.
Z01 HD 00637-03	Steroid Hormone Action in Female Reproduction Lynnette Nieman, M.D.
Z01 HD 00638-03	Physiology of Hypercortisolism Lynnette Nieman, M.D.
Z01 HD 00639-01	Genetics of Diabetes Mellitus Domenico Accili, M.D.
Z01 HD 00640-01	Developmental Endocrinology of Bone and Mineral Metabolism Jeffrey Baron, M.D.
Z01 HD 00641-01	Physiology and Genetics of Obesity J. Yanovski, M.D.

Z01 HD 00642-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00610-16 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Growth, Puberty, Their Disorders: Physiology, Pathophysiology, and Molecular Biology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G.B. Cutler	Head	SDE, DEB, NICHD
Others:	(see attached)		

COOPERATING UNITS *(if any)*

(see attached)

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Developmental Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.8	5.0	1.8

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
 ☒ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to advance understanding of the mechanisms that underlie normal and abnormal puberty, and to apply this knowledge to improve existing therapy for disorders of puberty. Since somatic growth is a major determinant of the timing of pubertal onset, a further objective is to clarify the mechanisms of normal growth and of growth failure. Principle areas of laboratory investigation include the mechanisms of normal growth and puberty. Principle areas of clinical investigation include the mechanism of premature thelarche and of the gonadotropin-independent forms of precocious puberty, the developmental changes in hypothalamic regulation of gonadotropin secretion, the behavioral changes associated with normal and abnormal pubertal development, the mechanisms of prepubertal and pubertal growth, the role of pubertal sex steroids in the acquisition of normal adult bone density, the treatment of central precocious puberty with an analog of luteinizing hormone-releasing hormone (LHRH), the treatment of familial male isosexual precocious puberty with combined antiandrogen and aromatase inhibitor, the evaluation of new approaches to the diagnosis of growth hormone deficiency and to the differential diagnosis of delayed puberty, the treatment with synthetic parathyroid hormone of children with hypoparathyroidism, the treatment with growth hormone of children with Turner syndrome and with non-growth hormone-deficient short stature, and the treatment with insulin-like growth factor-1 of children with growth hormone insensitivity and of children with non-growth hormone-deficient short stature.

The principal areas of laboratory investigation include the structure and function of the regulatory sequences of the human gonadotropin-releasing hormone (GnRH) gene, molecular analysis of the gene for the luteinizing hormone receptor in familial male precocious puberty and in Leydig cell hypoplasia, and of the gene for the calcium-sensing receptor in sporadic and autosomal dominant hypoparathyroidism.

PHS 6040 (Rev. 5/92)

Others:

K.M. Barnes	Chemist	SDE, DEB, NICHD
V. Abad	Clinical Associate	SDE, DEB, NICHD
J. Baron	Head, UGD	SDE, DEB, NICHD
D. Counts	Special Volunteer	SDE, DEB, NICHD
F. DeLuca	IRTA Fellow	SDE, DEB, NICHD
A. Cunningham	Pre-IRTA Fellow	SDE, DEB, NICHD
F. Czerwiec	Clinical Associate	SDE, DEB, NICHD
P. Feuillan	Special Volunteer	SDE, DEB, NICHD
P. Gore	Summer IRTA	SDE, DEB, NICHD
C. Heinrichs	Special Volunteer	SDE, DEB, NICHD
E. Leschek	Clinical Associate	SDE, DEB, NICHD
K. Oerter-Klein	Special Volunteer	SDE, DEB, NICHD
S. Rose	Special Volunteer	SDE, DEB, NICHD
J. Levine Ross	Special Volunteer	SDE, DEB, NICHD
J. Uyeda	Pre-IRTA Fellow	SDE, DEB, NICHD
A. Weinstein	Summer IRTA	SDE, DEB, NICHD
K. Winer	IRTA Fellow	SDE, DEB, NICHD
K. Yano	Visiting Associate	SDE, DEB, NICHD
J. Yanovski	Clinical Associate	SDE, DEB, NICHD

Cooperating Units

J. Jones, L. Long, D. Blum, S. Hill, A. Dwyer, T. Shawker, Clinical Center, NIH; M. Bolander, Mayo Clinic; A. Hidaki, M. Saji, L.D. Kohn, Laboratory of Biochemistry and Metabolism, NIDDK; W.-Y. Chan, S.-M. Wu, L. Blomberg, Department of Pediatrics, Georgetown University; A.J.W. Hsueh, M. Kudo, S.Y. Hsu, Department of Obstetrics and Gynecology, Stanford University Medical Center.

Project Description

Objectives:

We seek to increase understanding of normal and abnormal growth and pubertal development, and to apply this knowledge to improve treatment for disorders of growth and puberty. The specific objectives are:

1) to determine gonadotropin pulse frequency and amplitude during normal puberty, and to compare the normal pattern with the pattern in children with central precocious puberty, particularly children with a hypothalamic hamartoma;

2) to identify the molecular mechanism of testicular autonomy in boys with familial male precocious precocity (FMPP), and of Leydig cell failure in Leydig cell hypoplasia, through analysis of the gene for the luteinizing hormone (LH) receptor;

3) to explore the mechanism of premature thelarche;

4) to develop improved methods for the diagnosis of growth hormone deficiency and for the differential diagnosis of delayed puberty;

5) to determine the efficacy and safety of treating the gonadotropin-independent ovarian function of McCune-Albright syndrome with aromatase inhibitors;

6) to determine the efficacy and safety of treating familial male precocious puberty with combined antiandrogen and aromatase inhibitor;

7) to determine the efficacy and safety of LHRH analog therapy of central precocious puberty through long-term follow-up of children under treatment;

8) to understand the mechanism of the pubertal growth spurt and its relationship to prepubertal growth, to optimize medically-induced prepubertal and pubertal growth in Turner syndrome through clinical trials of growth hormone (with and without low-dose estrogen treatment), to understand the mechanisms of non-growth hormone (GH)- deficient short stature and to improve the long-term outcome, and to assess the efficacy and safety of LHRH agonist-induced pubertal delay, of growth hormone treatment, and of treatment with insulin-like growth factor-1 on adult height in children with non-GH-deficient short stature;

9) to improve understanding of the role of estrogen in growth and development during childhood, early puberty, and the pubertal growth spurt by applying a newly developed ultrasensitive recombinant cell bioassay for estradiol;

10) to explore the role of pubertal sex steroid secretion in the acquisition of normal adult bone density;

11) to study the structure and regulation of the normal human LHRH gene and to compare the observations with the results in genetic disorders causing early or late puberty; to study the regulation of the LHRH gene and of LHRH secretion in a cultured LHRH neuronal cell line;

12) to correct the absence of parathyroid hormone action on bone and kidney in patients with hypoparathyroidism by administration of synthetic parathyroid hormone;

13) to identify the molecular mechanism of autosomal dominant and sporadic hypoparathyroidism through analysis of the gene for the calcium-sensing receptor.

Methods Employed:

1) Hypothalamic-pituitary maturation during puberty-steroid and peptide hormone radioimmunoassay and bioassay.

2) Mechanism of familial male precocious puberty and of Leydig cell hypoplasia - molecular studies of the LH receptor gene by PCR, sequencing, site-directed mutagenesis, and expression in cultured cells.

3) Treatment of precocious puberty - clinical, radiographic, behavioral, and hormonal evaluation.

4) Mechanism of normal prepubertal and pubertal growth, and of idiopathic growth failure - bioassay and radioimmunoassay of growth factors; immunostaining of peptide growth factors in epiphyseal cartilage; in situ hybridization, solution hybridization, and/or Northern analysis to quantitate growth factor mRNAs in epiphyseal cartilage; infusion of growth factors or growth factor antagonists into the rabbit proximal tibial epiphyseal growth plate; creation of transgenic animals from embryonic stem cells with heterozygous or homozygous cartilage-specific deletion of growth factors; molecular studies of the growth hormone (GH) receptor (GHR) gene.

5) Treatment of growth failure - randomized, double-blind, placebo-controlled trials with adult height as the principal growth endpoint; short-term studies to define the conditions that appear optimal for long-term evaluation.

6) Mechanism of autosomal dominant or sporadic hypoparathyroidism - molecular studies of the calcium-sensing receptor gene by PCR, sequencing, site-directed mutagenesis, and expression in cultured cells.

Progress:

1. Mechanism and treatment of precocious puberty

a. Familial male precocious puberty (FMPP) and Leydig cell hypoplasia - molecular mechanism

In studies of the structural requirements for activating mutations of the LH receptor (LHR), eleven of the 12 activating mutations (8 of which were discovered in this laboratory) are in a 40-amino acid region from position 542 to 581, which spans the fifth and sixth transmembrane domains and the intervening third intracellular loop. This hot spot for activating mutations identifies a region that plays a critical role in constraining the unliganded receptor in an inactive conformation. Recently, the twelfth known activating LHR mutation, Met 398 Thr, has been discovered in a novel region involving the second helical transmembrane domain. We hypothesize that the wild-type methionine residue at 398 is involved in a hydrogen bond with one of the residues in the hot spot region, and that disruption of this bond by mutation either at 398 or in the hot spot region leads to constitutive activation. Computer analysis of the naturally occurring mutant LH/CG receptors (LHR) in patients with male-limited precocious puberty (MPP) suggested that Phe 576 is a critical bridging residue important for receptor activity. Despite this, no mutation at this residue causing MPP has been described. To analyze further the functional role of Phe 576, 4 amino acid substitutions of this site were constructed and studied *in vitro* (F576G, F576E, F576I, and F576Y). Cells transfected with F576I and F576G showed constitutive activation and a further response to hCG, analogous to the original FMPP mutation, D578G. These mutant receptors were predicted to lose their helical conformation in the vicinity of the mutation. By contrast, the F576E mutation, which was predicted to change the receptor conformation in the distal third intracellular and sixth transmembrane domains into a totally helical conformation, created a null receptor that did not bind hCG nor increase cyclic adenosine monophosphate (cAMP) nor inositol phosphate (IP) levels in response to hCG. These data suggest that Phe 576 plays an important role in the LHR with respect to cAMP and IP signalling and receptor conformation. Thus, the studies of this rare form of precocious puberty have pinpointed a region of the LH receptor that is critical for signal transduction and hence for the hormonal regulation of human reproduction.

Leydig cell hypoplasia is an autosomal recessive form of male pseudohermaphroditism. To test the hypothesis that this disorder results from inactivating mutations of the LH receptor, we performed studies similar to those described above for FMPP. To date several mutations have been identified that appear to explain this disorder:

(1) a nonsense mutation (A1635C) in the fifth transmembrane domain that produces a truncated receptor, with reduced number and affinity of receptors in transfected cells, and a complete lack of signal transduction when exposed to hCG, (2) a missense mutation (Ser616Tyr) in the seventh transmembrane domain that does not alter receptor affinity but markedly impairs hCG-induced cAMP production per binding site, and (3) deletion of exon 8 (hLHR - Δ exon 8). The exon 8-deleted allele encodes a receptor that could not be detected by ligand-binding assays, thus indicating an important role of the exon 8 polypeptide in LHR expression or ligand-binding. We hypothesize that, as larger numbers of these patients are studied, the inactivating mutations will help define 3-dimensional domains that are critical for ligand-binding, on the one hand, and signal transduction via G-proteins on the other. Since the signal transduction domains are likely to be conserved among the large family of G-protein-coupled receptors, study of this rare experiment of nature could shed light on this basic mechanism of hormone signal transduction.

b. Familial male precocious puberty-treatment with combined antiandrogen and aromatase inhibitor

A long-term pilot study is ongoing to test the hypothesis that the growth rate, bone maturation, and adult height of these boys can be normalized with the regimen of antiandrogen (spironolactone) aromatase inhibitor (testolactone),and LHRH analog (deslorelin [for those boys who develop secondary LHRH-dependent precocious puberty]). In 7 boys who have been treated for at least 6 years, growth rate and bone maturation have been suppressed to normal rates, and the predicted adult height has increased from 161.3 to 171.9 cm. The study is continuing to determine whether this gain in predicted height will be realized at final height, and to treat enough subjects to obtain generalizable results. We have also initiated a new project to improve this regimen through the use of a more potent aromatase inhibitor (CGS 16949A [fadrozole]).

c. McCune-Albright syndrome - treatment with the aromatase inhibitor testolactone

A long-term pilot study is ongoing to test the hypothesis that the aromatase inhibitor, testolactone, can improve the growth rate, bone maturation, and adult height of girls with this disorder. We have also initiated a new project to improve this regimen through the use of a more potent aromatase inhibitor (CGS16949A [fadrozole]).

d. LHRH analog therapy of central precocious puberty

Analysis of mean adult height (-1.1 SD) in the first 44 children to attain adult height or near-adult height (within 2 cm) indicates a highly significant improvement over pretreatment predicted height (-2.0 SD) but a clear shortfall compared to expected height based upon parental height (+ 0.1 SD). However, the first children treated had the greatest delay of treatment from onset of symptoms (> 3 years), and thus it would be premature to conclude that this is the best result that can be achieved. Continued follow up of those children who were treated later and are still growing will test our hypothesis that there will be an even greater improvement in the final height of these children.

2. Mechanism and optimization of prepubertal and pubertal growth

a. The role of estradiol in prepubertal and pubertal growth

To test the hypothesis that girls secrete more estrogen than do boys during the prepubertal years, we developed an ultrasensitive recombinant cell bioassay for estradiol, based upon yeast cells genetically engineered for extreme sensitivity to estrogen, with a sensitivity of 0.02 pg/mL, approximately 100-fold more sensitive than previous radioimmunoassays. With this assay, we showed that estradiol levels in prepubertal girls (0.6 ± 0.6 pg/mL) are significantly higher than the level in boys (0.08 ± 0.2 pg/mL). This may explain the more rapid bone maturation, the earlier entry into puberty, and the earlier cessation of growth of girls compared to boys.

We have also used this assay to evaluate estradiol suppression by a new aromatase inhibitor (letrozole) in postmenopausal women with breast cancer. Letrozole, at the dose of 100 ug, reduced estradiol from 1.95 to 0.07 pg/mL, suggesting that this drug should prove useful in the treatment of estrogen-dependent conditions. From a broader perspective, this study suggests the potential range of applications of this new assay method. Indeed, in an editorial in the Journal of Clinical Investigation accompanying this publication, Dr. Jean Wilson stated that it should lead to a "renaissance in estrogen physiology." We are currently applying this assay to help assess the effectiveness of aromatase inhibitor treatment in children with congenital adrenal hyperplasia and in boys with familial male precocious puberty.

b. Treatment of Turner syndrome

Based upon the results of our earlier short-term studies, a long-term, randomized, double-blind, placebo-controlled clinical trial has been initiated to assess the effect on adult height, behavior, and cognitive function of estrogen, growth hormone, and combined estrogen and growth hormone treatment. The project has been organized as a two-site project, with 1/3 of the patients being followed at Thomas Jefferson University and 2/3 at the NIH, under a joint venture agreement with Eli Lilly and Company, which provides biosynthetic human growth hormone and certain additional support for the study. Approximately 150 patients out of a projected total of 160 patients have been enrolled in the study.

Although the major endpoints of this study remain blinded until the study conclusion, analysis of certain baseline and interim data has revealed several new findings. First, questionnaires from both the parents and the girls with Turner syndrome revealed weaker social relationships, school performance, and self-esteem compared to control girls matched for age, socioeconomic status, and verbal IQ. However, with administration of estrogen beginning at age 12, we found improved self-concept, both by self- and parental report, in girls with Turner syndrome followed longitudinally through adolescence, so that the girls with Turner syndrome resembled the normal controls by age 14-15. These findings underscore the need to initiate estrogen replacement therapy by ages 12-14 years in this population. Second, cognitive tests of the Turner girls has revealed a characteristic profile of impaired performance on tests of spatial recall, recognition, and reasoning (representing in magnitude about 1 SD unit relative to the control population). This cognitive profile appeared quite consistent from childhood through adolescence. Future analyses will determine the effect of sex steroid administration on this cognitive phenotype, and will examine the cognitive phenotype in girls with unusual karyotypes involving partial deletion of one X-chromosome in order to determine the relationship between the specific X-chromosomal region deleted and the cognitive phenotype. Thus, the early findings from this trial have revealed new behavioral and cognitive features of the Turner syndrome.

c. Mechanism of non-GH-deficient short stature

We previously published, in the New England Journal of Medicine, a comparison of diagnostic methods for growth hormone (GH) deficiency in 54 short children. The study found low sensitivity and diagnostic accuracy of the mean spontaneous 24-hour GH level compared to the peak GH level during 3 GH stimulation tests.

This original study involved only prepubertal children. More recently, we have extended our studies of spontaneous GH secretion (now measured as the mean 12-hour nighttime GH level) to include a large cohort of short children in each of the 5 stages of puberty. As in the original study of prepubertal short children, we observed a very low incidence of abnormally low mean nighttime GH levels among pubertal children with idiopathic short stature. When body mass index (which is inversely related to mean GH level) was taken into account, the incidence of low spontaneous growth hormone levels was not significantly greater than that in normal control subjects. Thus, low spontaneous GH secretion is a highly unusual cause of growth failure in pubertal, as in prepubertal, short children, and the routine use of spontaneous GH measurement to detect GH neurosecretory dysfunction in such children is unwarranted.

To test the hypothesis that the diagnostic usefulness of spontaneous growth hormone (GH) measurements could be improved by conducting measurements under circumstances that augment GH secretion, we propose to conduct these measurements during fasting and after the brief administration of sex steroid. We hypothesize that these measures will augment spontaneous GH levels in non-GH-deficient short stature but not in true GH deficiency, thus giving rise to a clinically useful test for patients in whom the GH stimulation tests yield equivocal results.

d. Mechanism of growth failure in pediatric HIV infection

The AIDS epidemic has added pediatric HIV infection to causes of growth failure in children. The mechanism of growth failure in children with early HIV infection, however, remains unclear. To study thyroid function in HIV-infected children and to determine whether there are correlates of thyroid dysfunction with disease progression, we examined measures of thyroid function in 167 children with HIV infection. Free thyroxine levels were at or below the lower limit of normal in 18% of children, and thyrotropin and thyroxine-binding globulin (TBG) were above the normal range in 30%. Both TBG and thyrotropin correlated inversely with the CD4+ cells count ($r = -0.5$, $p < 0.01$ and $r = -0.23$, $p < 0.05$, respectively). These findings indicate that thyroid abnormalities occur more frequently in children with HIV than was previously reported, have a different profile from the thyroid abnormalities associated with other chronic disease conditions, and correlate with disease progression.

e. Treatment of non-GH-deficient short stature

1) Growth hormone

Recent studies from several centers have shown that biosynthetic human growth hormone (hGH) can increase short-term growth rate in non-GH-deficient short stature. However, short-term effects are not always sustained, and treatment-induced acceleration of pubertal onset could negate short-term gains. Moreover, the increasing off-label use of hGH for this purpose has given an important public health significance to the determination of whether such use is effective and safe. Thus, to address the long-term outcome of such treatment, a randomized, double-blind, placebo-controlled trial has been designed to determine the effect of biosynthetic human growth hormone on the adult height of children with non-GH-deficient short stature. A joint venture agreement with Eli Lilly and Company has been established to provide growth hormone and additional support for the study. Approximately 56 of the total of 80 subjects have been enrolled in this study.

We remain blinded to the major endpoints of the trial.

2) LHRH analog-induced pubertal delay

The hypothesis that adult height can be increased by prolonging the growth period through delay of puberty is supported by our observation of greater adult stature in patients with isolated hypogonadotropic hypogonadism (IHH) compared to normal subjects. To determine whether similar gains can be achieved through pharmacologic delay of puberty by LHRH analog, a randomized, placebo-controlled trial was initiated that is now fully enrolled (50 subjects). The preliminary results of this trial are now available in the first 37 subjects who have attained adult height. The adult height of the LHRH agonist-treated subjects was 6.8 cm (2.7 inches) greater than that of the randomized, placebo control group ($p < 0.001$). This is the first randomized trial to show that delay of a normally timed puberty (by 3.6 years) can increase adult height significantly in children with non-GH-deficient short stature.

3) Insulin-like growth factor-1

Recombinant human IGF-I, the growth factor believed to mediate the growth-promoting effects of GH, has become available for clinical investigation. We have initiated a protocol to test the hypothesis that this agent will stimulate growth both in patients with Laron dwarfism, who are resistant to growth hormone due to an absence of functional growth hormone receptor, and in children with non-GH-deficient short stature, who have variable responses to growth hormone that in some cases resemble those of Laron dwarfs.

Since the metabolic effects of growth hormone and IGF-1 on glucose metabolism act in opposing directions, we hypothesize that the combination of these agents might have fewer adverse metabolic effects than either agent alone. Additionally, evidence for synergistic effects of growth hormone and IGF-1 in several systems in vitro suggests the hypothesis that they also may have synergistic effects upon epiphyseal growth in vivo. Thus, we envision, once the effects of IGF-1 alone have been determined, additional studies to test the hypothesis of a synergistic effect of these agents upon linear growth.

3. Differential diagnosis of delayed puberty

No satisfactory diagnostic test is available to determine whether a child with delayed puberty has hypogonadotropic hypogonadism and will never enter puberty spontaneously or has constitutional delay of puberty and will eventually enter puberty on his own. The recent observation that cholecystokinin can stimulate LHRH secretion suggests the hypothesis that children with hypogonadotropic hypogonadism, who lack normally functioning LHRH neurons, will fail to respond to cholecystokinin, whereas children with constitutional delay of puberty will respond normally.

4. Treatment and molecular mechanism of hypoparathyroidism

To test the hypothesis that synthetic parathyroid hormone 1-34 (PTH) could maintain normal serum calcium in hypoparathyroid patients with reduced urine calcium compared to conventional treatment with calcitriol, Karen Winer performed a randomized, cross-over trial comparing these two agents. As we had predicted, PTH permitted equivalent control of serum calcium while achieving a significant reduction in urine calcium. These findings have been accepted for publication in the *Journal of the American Medical Association.*

In a second study to define the optimal dose regimen for PTH, Dr. Winer compared the outcome of once-daily vs. twice-daily PTH therapy. The twice-daily regimen allowed serum calcium to be maintained within the target therapeutic range with a significantly reduced total daily dose (40 ± 6 vs. 83 ± 11 μg/d, $p < 0.0001$), and also resulted in reduced amplitude of serum calcium excursions and a reduced rate of bone turnover as reflected by markers of bone formation and bone resorption. We concluded that twice-daily, compared to once-daily, PTH treatment of hypoparathyroidism provides more physiologic regulation of calcium and bone metabolism at a reduced total PTH dose. Based upon these findings, we have extended this pilot study to test the hypothesis that twice-daily PTH treatment will reduce the incidence of renal complications (such as nephrolithiasis, nephrocalcinosis, and decreased renal function) in this disorder.

In the course of this clinical trial, we received referrals of several families with autosomal dominant hypoparathyroidism. A noteworthy feature of these families was that they all had impressive hypercalciuria, even when serum calcium was below the normal range. Following the cloning of the calcium-sensing receptor in December 1993, we hypothesized, based upon our studies of familial male precocious puberty, that the mechanism of this disorder would be an activating mutation of the calcium-sensing receptor. Jeffrey Baron and coworkers showed this to be the case. Two families had mutations in or immediately adjacent to the transmembrane domain region, which presumably activate signal transduction. Another family had a mutation in the extracellular domain, where it may act to increase the receptor affinity for calcium, based upon a reduced EC_{50} for receptor activation by calcium *in vitro*. Additionally, we found de novo activating mutations of the calcium-sensing receptor in two children with sporadic hypoparathyroidism and marked hypercalciuria.

The activating mutations of the calcium-sensing receptor predicted a previously unrecognized phenotype of marked hypercalciuria in autosomal dominant hypoparathyroidism. Since the calcium-sensing receptor is expressed in kidney, where it is believed to negatively regulate calcium reabsorption, an activating mutation of this receptor would be expected to cause hypercalciuria even when serum calcium was normal or low. Direct comparison of urine calcium in patients with inherited and acquired hypoparathyroidism showed that the patients with the autosomal dominantly inherited form had significantly greater urine calcium excretion at any given serum calcium level. Moreover, they did not reduce urine calcium as serum calcium decreased, in contrast to the patients with acquired hypoparathyroidism.

Significance to Biomedical Research and the Programs of the Institute:

Growth and puberty are fundamental developmental processes. Growth failure (stature more than 2 standard deviations below the normal height for age) affects approximately 1.5 million American children below the age of 18. Severe growth failure (stature more than 3 standard deviations below normal) affects approximately 100,000 children. Particularly for this severely short group, growth failure may have adverse consequences for the individual's psychological, social, educational, and career development. Thus, improved understanding and treatment of growth failure would benefit a substantial number of children in this country and throughout the world. Additionally, progress in understanding the molecular mechanisms of epiphyseal growth may have implications for understanding the fundamental processes of growth and differentiation in other tissues. Finally, the production of both human growth hormone and insulin-like growth factor-1 by recombinant DNA technology will continue to cause an increasing empiric use of these and other growth-promoting agents in an attempt to increase the adult height of children with growth failure. Thus, the safety and efficacy of growth-promoting agents in this setting have become a public health issue that requires controlled clinical trials.

Precocious puberty occurs in approximately 1 in 5,000 children and often has a profound physical and emotional impact on the affected individuals. The above studies have been highly successful in controlling central and peripheral precocious puberty, disorders for which there has previously been no effective treatment. Progress is also being made in understanding the pathophysiology of the McCune-Albright syndrome and familial male precocious puberty, and in understanding normal human pubertal physiology. Additionally, children with precocious puberty have provided a unique model in which to analyze the influence of hormonal, somatic, and social-environmental factors on the behavioral changes of puberty, which are of importance both to the individual and to society.

Hypoparathyroidism is a rare but potentially fatal disorder that may be acquired, through autoimmune destruction or inadvertent surgical removal during thyroid or parathyroid surgery, or inherited. Although rare, hypoparathyroidism represents a unique opportunity for the scientific study of the role of parathyroid hormone in bone and calcium metabolism. The knowledge gained through such study may have implications beyond this particular disorder, such as to patients with osteoporosis or other metabolic bone disorders. These other disorders represent a major public health problem for which improved understanding of parathyroid hormone effects on bone could be of therapeutic importance.

Proposed Course:

1. The molecular mechanism of familial male precocious puberty (FMPP) and Leydig cell hypoplasia

a. DNA will be obtained from additional affected kindreds. PCR and DNA sequencing of the LH receptor gene will be performed to determine which mutations other than those that are currently known can give rise to these disorders, since each new mutation provides information with which to construct an increasingly precise model of the structural basis for LH receptor activation.

2. Treatment of peripheral precocious puberty

a. Children receiving testolactone treatment of McCune-Albright syndrome and combined testolactone/spironolactone treatment of familial male precocious puberty will be followed until adult height has been achieved. Such follow-up is essential to determine whether the apparent early benefits of treatment will be sustained and whether any significant adverse effects will emerge.

b. Improved therapy for peripheral precocious puberty - the aromatase inhibitor (testolactone) employed in our studies was chosen because of its excellent long-term safety record. Testolactone, however, is not as potent an aromatase inhibitor as would be desirable, and it is very expensive (average retail cost of $7,000 per year at the dose employed). More potent aromatase inhibitors are undergoing clinical development. Thus, new patients with FMPP will be randomized to receive fadrozole (CGS 16949A) or testolactone in a crossover design, since fadrozole is approximately 500 times more potent than testolactone. The efficacy and safety of fadrozole will also be evaluated in girls with McCune-Albright syndrome.

Additionally, our long-range objective is to intercept the pathophysiology of FMPP at the molecular level. Improved knowledge of mechanism could lead to therapies directed at preventing gonadal activation, such as gene therapy or the development of inverse LH agonists capable of restoring basal function in constitutively activated LH receptors, rather than the current strategy of blocking the consequences of gonadal activation.

3. LHRH analog therapy of central precocious puberty

The children currently receiving treatment will be seen annually or biannually until age 11 or 12, at which time treatment will be discontinued. Thereafter they will be seen annually (annual follow-up will be used for the subset of patients who are of greatest research interest because of long duration of treatment and/or proximity to attaining adult height) or biannually until final height is achieved. We plan to maintain an up-to-date address register that will permit subsequent assessment of reproductive function and normalcy of offspring.

4. Optimization of prepubertal and pubertal growth

a. Turner syndrome

We will continue the long-term, randomized, double-blind, placebo-controlled trial to assess the effect on adult height, behavior, and cognitive function of low-dose estrogen, growth hormone, and the combination of estrogen and growth hormone.

b. Pubertal delay as a treatment to enhance adult height

The ongoing randomized, double-blind trial of LHRH analog compared to placebo will be continued. The trial is fully enrolled (50 subjects), and the children are now being followed until adult height is achieved.

c. Pharmacologic growth hormone treatment in non-growth hormone-deficient in short stature

We will continue the long-term, randomized, double-blind, placebo-controlled trial of the effect of growth hormone on adult height in children with non-growth hormone-deficient short stature.

d. The role of growth factors in epiphyseal growth

We will evaluate whether or not the growth in children with GH insensitivity and with non-GH-deficient short stature can be stimulated with recombinant human insulin-like growth factor-1 (rhIGF-1).

The potential importance of direct administration of growth factors, compared to administration of growth hormone (GH) to stimulate growth factors endogenously, is three-fold. First, it may provide a new approach to treatment of patients with Laron dwarfism, who have resistance to GH and do not respond to GH treatment. It may also be useful in treating patients with familial deletion of the GH gene, who lack tolerance to GH and develop growth-attenuating antibodies to GH during GH therapy. Secondly, we postulate that there may be negative feedback events that restrain the response to exogenous GH. We hypothesize that rhIGF-1 may bypass such regulatory events, permitting greater responses than could be achieved through GH administration. Additionally, there is evidence in several settings of synergistic interaction between GH and IGF-1. Thirdly, the effects of GH and IGF-1 on glucose metabolism are opposite, which suggests the hypothesis that the combination of these agents may produce less alteration of glucose homeostasis than either agent alone.

5. Improved differential diagnosis of delayed puberty

Since cholecystokinin has been shown recently to stimulate LHRH release, we will study whether cholecystokinin can be used to assess LHRH secretion in man. The test will be used to examine the hypothesis that idiopathic hypogonadotropic hypogonadal patients will not respond to cholecystokinin, whereas subjects with constitutional delay of puberty will respond.

6. Treatment of hypoparathyroidism

We have conducted a pilot study to assess the hypothesis that calcium metabolism in hypoparathyroidism can be normalized with once-daily or twice-daily subcutaneous injections of synthetic parathyroid hormone. The results of the initial phase of the study have confirmed this hypothesis. A short-term, follow-up study has indicated improved efficacy and safety with the twice-daily PTH regimen. We are currently conducting a long-term randomized, parallel study to test the hypothesis that twice-daily PTH treatment can reduce the incidence of renal complications compared to calcitriol treatment.

Protocols:

Human

79-CH-112 Cutler, Barnes Treatment of true precocious puberty with a long-acting luteinizing hormone releasing factor analog

This study tests the hypothesis that the growth and pubertal development of children with LHRH-dependent precocious puberty can be normalized by treatment with deslorelin, a long-acting LHRH analog. The study employs an open design in which the major outcomes are compared with the subjects' pre-treatment baseline values (for example, final adult height is compared with predicted height before treatment and hormone levels during treatment are compared with the levels before treatment). The final height data are also being compared with the subjects' target height, the height of the normal population, and the height of untreated historical controls from the literature.

The current results indicate that deslorelin treatment reduces sex steroid levels to near prepubertal values, decreases growth rate and the rate of bone maturation, and produces final heights that are intermediate between the pretreatment predicted height and the children's target height based upon their genetic background. We conclude that the treatment is effective. Observation is ongoing to determine the adult height of those children who were recognized and treated earliest (who may have a better outcome), to assess the children's reproductive outcome (and thus the reproductive toxicity, if any), and to suppress secondary LHRH-dependent precocious puberty in children who are participating in treatment trials for McCune-Albright syndrome, familial male precious puberty, and congenital adrenal hyperplasia (see below).

82-CH-165 Feuillan, Jones, Nunez Testolactone treatment of girls with LHRH analog-resistant precocious puberty due to autonomous, non-neoplastic ovarian estrogen secretion

This study tests the hypothesis that the growth and development of girls with the McCune-Albright syndrome can be normalized by treatment with the aromatase inhibitor, testolactone. The long-term portion of the study employs an open design in which the outcome measures are compared with the subjects' pretreatment baseline values and the normative values for control subjects.

The results to date indicate substantial improvement in the majority of subjects, with decreased frequency or cessation of menses and decreased rates of growth and bone maturation. Some patients appear to escape from the effects of treatment after being effectively controlled for 2 to 3 years. This escape is not attributable to secondary central precocious puberty. We conclude that testolactone is a safe and effective treatment for the precocious puberty of McCune-Albright syndrome; however, normalization of growth and development have not yet been achieved, and more effective therapy is needed.

83-CH-199 Yanovski, Jones, Rose A double-blind, randomized, placebo-controlled clinical trial of luteinizing hormone releasing hormone analog (LHRH A) in pubertal patients with extreme short stature

This study tests the hypothesis that delay of puberty for 4 years by treatment with the LHRH analog, deslorelin, will increase the adult height of children with non-GH-deficient short stature and a normally timed puberty. The design is a randomized, double-blinded, placebo-controlled clinical trial. All 50 subjects have been enrolled.

Final outcome measures are not available because the trial is ongoing. A preliminary, interim analysis of the first 37 children to have attained adult height showed a significant increase in the height of the LHRH analog-treated subjects by 6.8 cm compared to the randomized, placebo-treated control subjects. We conclude that pubertal delay induced by deslorelin significantly increases predicted adult height in adolescents with short stature and a normally timed puberty.

91-CH-46	Cutler, Baron, Rose, Yanovski, Winer, Ross, Cassorla	A randomized, double-blind, placebo-controlled clinical trial of the effects of growth hormone therapy on the adult height of non-growth hormone-deficient children with short stature

This trial tests the hypothesis that supplemental GH administration will increase the adult height of children with non-GH-deficient short stature, without adverse effects owing to supraphysiologic GH dosage. The fundamental rationale for the study is to protect the public health, since thousands of non-GH-deficient children are currently receiving GH treatment before there is convincing evidence of its safety or efficacy in this setting. The design is a randomized, placebo-controlled, clinical trial. Approximately 3/4 of the 80 subjects have been enrolled.

Results of the major endpoints of the study are unavailable because the trial is ongoing. However, the hypothesis that GH decreases thyroid function in some subjects with non-GH-deficient short stature has been examined through a grouped analysis, by an independent statistician, in a manner which preserved the double-blind study design. This analysis showed that supplemental GH, at the dose and frequency employed, did not alter thyroid function during 12 months of treatment in these subjects.

85-CH-16	Cutler, Leschek, Jones	Spironolactone and testolactone treatment of boys with familial male precocious puberty

Total accrual approved: 50
Accrual to date: 30
Project start date: 1985
Projected finish date: 2010

This study tests the hypothesis that the growth and development of boys with familial male precocious puberty can be normalized by the combination of an antiandrogen, spironolactone, and an aromatase inhibitor, testolactone. The study employs an open design in which the major outcomes are compared with the subjects' pretreatment baseline values and, in the case of growth, with the published standards for the normal population.

The current results, from the initial pilot study, show that the regimen is effective in controlling growth, bone maturation, and pubertal development until secondary LHRH-dependent precocious puberty ensues. At that point, addition of the LHRH analog, deslorelin, can maintain effective control. However, further study is needed to determine the final adult height in the treated patients.

87-CH-152	Cutler, Ross	A double-blind, randomized, placebo-controlled trial of the effect of biosynthetic growth hormone and/or ethinyl estradiol on adult height in patients with Turner syndrome

This trial tests the hypothesis that supplemental GH treatment, with or without ultra-low, replacement doses of ethinyl estradiol, will increase the adult height of girls with Turner syndrome without adverse effects attributable to the drug regimen. The design is a 4-arm, randomized, placebo-controlled clinical trial, to adult height. The four arms are GH, early (age 5-12) low-dose ethinyl estradiol, GH plus early low-dose ethinyl estradiol, and a double placebo. The starting ethinyl estradiol doses from age 5-8 are 25 ng/kg/d and from age 8-12 are 50 ng/kg/d. However, in recognition of the fact that some girls with Turner syndrome undergo spontaneous puberty, and that sensitivity to estrogen varies among subjects, the protocol provides for dosage decreases of 50% whenever there is breast development below the age of 12 or bone advancement of 1 year within a 6-month period. The dosage can be further decreased by 50% decrements (to 25% or 12.5% of the original dose) if breast development or bone age acceleration persist. Thus, the actual estrogen doses during the prepubertal years in these subjects are extremely small, and are intended to approximate the prepubertal estrogen secretion rate of normal girls. After age 12, all subjects receive the same estrogen regimen beginning at the relatively low dose of 100 ng/kg/day from age 12 to 14, and doubling annually thereafter until menses occur. Approximately 150 of the total 160 subjects have been enrolled.

Major outcome measures from the trial are unavailable because the trial is ongoing. However, analysis of the baseline data from 137 girls with Turner syndrome, before estrogen or growth hormone treatment, revealed significantly increased cholesterol levels after age 11 compared to control girls (195 ± 38 vs. 165 ± 26 mg/dL, $p < 0.01$), which remained elevated after adjustment for body mass index. Secondly, cognitive tests of the Turner girls have revealed a characteristic profile of impaired performance on tests of spatial recall, recognition, and reasoning (representing in magnitude about 1 SD unit relative to the control population), which appeared quite consistent from childhood through adolescence. Thirdly, questionnaires from both the parents and the girls with Turner syndrome revealed weaker social relationships, school performance, and self-esteem compared to control girls matched for age, socioeconomic status, and verbal IQ. With administration of estrogen beginning at age 12, however, we observed improved self-concept, both by self- and parental report, so that by age 14-15 the girls with Turner syndrome resembled the normal controls. This behavioral improvement was not observed in a comparison group of girls with Turner syndrome in whom estrogen treatment was delayed. Thus, the early findings from this trial have revealed new metabolic, cognitive, and behavioral features of the Turner syndrome.

This trial, and the Canadian Turner syndrome GH trial, are the only ongoing studies in the world in which the effect of GH in Turner syndrome is being compared to an untreated control group. Thus, the trial will play an important role in resolving this important clinical issue.

90-CH-123	Cutler, Ross	Estrogen effects on cognition in girls with Turner syndrome

This study tests the hypothesis that the cognitive phenotype of Turner syndrome, namely, decreased performance on tests of spatial recognition, reasoning, and recall, is attributable to estrogen deficiency and will therefore be correctable, at least in part, by estrogen replacement. The study is a companion protocol to project 87-CH-152 in the sense that most of its subjects are also taking part in that study.

Results from this study are not available because of the blinded nature of protocol 87-CH-152. However, the baseline, pretreatment data have confirmed the characteristic features of the Turner cognitive phenotype and have confirmed its stability over time when compared to an untreated adolescent-aged cohort of Turner girls who are not taking part in 87-CH-152. The randomized, placebo-controlled design of this study provides an ideal circumstance in which to test the hypothesis of sex steroid effects on cognitive function, since there is greater control of other variables than has been possible in previous studies.

90-CH-179	Nunez	CGS 16949A treatment of girls with precocious puberty due to gonadotropin-independent ovarian estrogen secretion

This pilot study tests the hypothesis that the new aromatase inhibitor, CGS 16949A or fadrozole, can control the precocious puberty of the McCune-Albright syndrome. The study employs an open design in which the outcome measures during 6 months before treatment are compared with those during treatment. Long-term outcomes, such as adult height, will be compared with the pretreatment predicted adult height, the target height (determined from parental height) and the normal height of the population.

Current results show incomplete blockade of estrogen production at the dose 240 of ug/kg/day, with improved suppression of menses at the higher dose of 480 ug/kg/day. Since even the higher dose does not completely suppress menses in all affected girls, we plan to test the effectiveness of even more potent aromatase inhibitors once they are available for pediatric use.

91-CH-147	Cutler, Leschek	Dose-response study and placebo-controlled crossover trial of the effects of IGF-1 therapy on growth velocity in children with GH insensitivity and in children with non-GH-deficient short stature

Total accrual approved: 50 (40 non-growth hormone-deficient short stature, 10 growth hormone-deficient short stature)
Accrual to date: 22 (17 non-growth hormone-deficient short stature, 5 growth hormone-deficient short

stature)
Project start date: 1991
Projected finish date: 2000

This study tests the hypothesis that exogenous IGF-I administration can increase the growth velocity of children with GH insensitivity or with other forms of non-GH-deficient short stature. The study employs a double-blind, randomized crossover design. Results of the study are not available because the trial is ongoing.

91-CH-83 Cutler A randomized clinical trial of luteinizing hormone-releasing hormone analog in pubertal patients with isolated growth hormone deficiency treated with growth hormone

This study tests the hypothesis that depot leuprolide-induced pubertal delay will increase the adult height of GH-treated children with classical GH deficiency. The study employs a randomized, open design - all of the children receive GH, and approximately one-half are randomized to receive also the LHRH analog, depot leuprolide. Results are not available since the trial is ongoing and few subjects have attained adult height.

92-CH-011 Winer Treatment of hypoparathyroidism with synthetic human parathyroid hormone 1-34

This study tests the hypothesis that once-daily synthetic parathyroid hormone 1-34 (PTH) will normalize serum calcium and reduce the incidence of hypercalciuria, compared to conventional treatment with calcitriol, in patients with hypoparathyroidism. The study employs a randomized, open, crossover design lasting 10 weeks for each arm for the initial dose-adjustment, feasibility phase. Following this initial phase, a second study is testing the hypothesis that twice-daily PTH treatment will normalize calcium metabolism better than once-daily treatment. This second study also employs a randomized, open, crossover design lasting 10 weeks for each arm. Lastly, a long-term study will test the hypothesis that twice-daily PTH will reduce the incidence of renal complications compared to twice-daily calcitriol. This study will employ a randomized, open, parallel design with renal complications (decreased glomerular filtration rate, nephrolithiasis, and nephrocalcinosis) as the major outcome measures.

The current results indicate that PTH, administered once-daily, normalizes serum calcium as well as twice-daily calcitriol while producing significantly lower urine calcium levels at any given level of serum calcium. The preliminary results of twice-daily PTH administration indicate that it normalized calcium metabolism more effectively than once-daily administration. We conclude that PTH offers better short-term control of calcium metabolism than calcitriol in patients with hypoparathyroidism. The effect of long-term PTH administration will require further study.

93-CH-0054 Cutler, Ross The relative effects of androgen, estrogen, and the combination of androgen and estrogen on growth rate, GH binding protein, IGF-1, and cognitive function in Turner syndrome

This study tests the hypothesis that androgen and estrogen treatment will improve the cognitive function of girls with Turner syndrome in tests of spatial recognition, reasoning, and recall. Additionally, we hypothesize that androgen treatment will have a greater effect on this aspect of cognitive function than estrogen treatment. Lastly, the study tests the hypothesis that androgen and estrogen will have additive effects on growth velocity.

The study employs a randomized, four-arm, open design, with an enrollment goal of 80 subjects. No results are available since the study is still ongoing.

The study employs a randomized, open design in which the patient receives 2 IPSS procedures, one with and one without prior 8 mg per day dexamethasone administration for 2 days. The diagnostic performance of the conventional and modified IPSS procedure will then be compared against the "gold standard" diagnosis determined at follow-up (the "gold standard" is surgical cure or pathologic diagnosis for Cushing syndrome and remission or prolonged lack of progression for pseudo-Cushing states).

Preliminary results suggest that the Dex/IPSS procedure has improved diagnostic accuracy compared to IPSS in settings in which normal corticotroph function may not be suppressed. We conclude that further evaluation of this new test is warranted.

94-CH-104	Leschek	An open, randomized clinical trial of spironolactone and fadrozole vs. spironolactone and testolactone treatment of boys with familial male-limited precocious puberty (FMPP)

This study tests the hypothesis that the new, more potent aromatase inhibitor, fadrozole, when combined with the antiandrogen, spironolactone, will improve the control of growth and bone maturation in boys with FMPP. The study employs a randomized, crossover design in which the major outcomes of a 6-month trial of the new regimen are compared to the same outcomes after a 6-month trial of testolactone and spironolactone (with a 3-month washout period between the arms). No results are available because the trial is ongoing.

Publications:

Baron J, Winer KK, Yanovski JA, Cunningham AW, Laue L, Zimmerman D, Cutler GB Jr. Mutations in the Ca^{2+}-sensing receptor gene cause autosomal dominant and sporadic hypoparathyroidism. Hum Mol Genet 1996;5:601-6.

Chan WY, Cutler GB Jr. Molecular aspects of precocious puberty. Adv Mol Cell Endocrinol, in press.

Cutler GB Jr. Central puberty in disorders with accelerated peripheral maturation. In: Plant, T.M. and Lee, P.A., eds. The neurobiology of puberty. Bristol, J Endocrinol Ltd 1995;209-15.

Cutler GB Jr. Precocious puberty. In: Hurst, J.E., ed. Medicine for the Practicing Physician, 4th ed. Stamford, Connecticut: Appleton Lange 1996;623-8.

Hirschfeld S, Laue L, Cutler GB Jr., Pizzo PA. Thyroid abnormalities in children infected with human immunodeficiency virus. J Pediatr 1996;128:704.

Klein KO, Cutler GB Jr. Puberty. In: Hillier SG, Kitchener H, Nielson JP, eds. Scientific essentials of reproductive medicine. London: W.B. Saunders, 1996;196-207.

Klein KO, Demers LM, Santner SJ, Baron J, Cutler GB Jr, Santen RJ. Use of ultrasensitive recombinant cell bioassay to measure estrogen levels in women with breast cancer receiving the aromatase inhibitor, letrozole, J Clin Endocrinol Metab 1995;80:2658-60.

Laue L, Wu SM, Kudo M, Bourdony CJ, Cutler GB Jr., Hsueh AJW, Chan WY. Compound heterozygous mutations of the luteinizing hormone receptor gene in Leydig cell hypoplasia. Mol Endocrinol 1996;10:987-97.

Leschek EW, Doppman JL, Pass HI, Cutler GB Jr. Clinical Case Seminar: Localization by venous sampling of occult chorionic gonadotropin-secreting tumor in a boy with mosaic Klinefelter syndrome and precocious puberty. J Clin Endocrinol Metab, in press.

Nunez SB, Blye RP, Thomas PM, Reel JR, Barnes KM, Malley JD, Cutler GB Jr. Recovery of reproductive function in male and female rats treated with the potent aromatase inhibitor fadrozole hydrochloride. Reprod Toxicol, in press.

Rose SR, Municchi G, Barnes KM, Cassorla FG, Cutler GB Jr. Overnight growth hormone concentrations are usually normal in pubertal children with idiopathic short stature - a clinical research center study. J Clin Endocrinol Metab 1996;81:1063-8.

Ross JL, McCauley E, Roeltgen, Long L, Kushner H, Feuillan P, Cutler GB Jr. Self-concept and behavior in adolescent girls with Turner syndrome: potential estrogen effects. J Clin Endocrinol Metab 1996;81:926-31.

Ross JL, Roeltgen D, Cutler GB Jr. The neurodevelopmental transition between childhood and adolescence in girls with Turner syndrome. In: Albertsson-Wikland, K. ed. Turner Syndrome in a life span perspective: research and clinical aspects. Amsterdam: Elsevier Press, 1995;297-308.

Winer KK, Yanovski JA, Cutler GB Jr. A short-term randomized, crossover trial of human parathyroid hormone 1-34 versus calcitriol in the treatment of hypoparathyroidism. J Am Med Assoc, in press.

Yano J, Kohn LD, Saji M, Kataoka N, Okuno A, Cutler GB Jr. A case of male-limited precocious puberty caused by a point mutation in the second transmembrane domain of the luteinizing hormone/choriogonadotropin receptor gene. Biochem Biophys Res Commun 1996;220:1036-42.

Yanovski JA, Cutler GB Jr. The reproductive axis: pubertal activation. In: Adashi EY, Rock JA, and Rosenwaks, Z, eds.: Reproductive endocrinology, surgery, and technology. New York, Raven Press, 1996;196-207.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00615-16 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Endocrine-Immune Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	G.P. Chrousos	Head	SPE, DEB, NICHD
Others:	See attached list:		

COOPERATING UNITS *(if any)*

P.W. Gold, E. Sternberg, Clinical Neuroendocrinology Branch, NIMH; R. Wilder, Arthritis and Rheumatism Branch, NIAMS; Gabriel Panayi, University of London, London UK; M.A. Magiakou and G. Mastorakos, Athens University Medical School, Athens, Greece; Theoharis Theoharides, Tufts University, Boston, MA.

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Pediatric Endocrinology

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.8	1.6	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to increase our understanding of the interactions between the endocrine and immune systems in both experimental animals and humans. Several immune system products, such as the inflammatory cytokines, Tumor Necrosis Factor-α, Interleukin-1, and Interleukin-6 activate the hypothalamic-pituitary-adrenal (HPA) axis and through it suppress and restrain the inflammatory/immune response. Interleukin-6 is particularly potent in humans, stimulating not only ACTH and cortisol but also arginine-vasopressin (AVP) secretion. Plasma interleukin-6 is elevated in glucocorticoid deficiency states and after exercise. Elevations of interleukin 6 in infectious, inflammatory, and traumatic states may explain the pathogenesis of the Syndrome of Inappropriate AVP Secretion observed in these states. We recently demonstrated that corticotropin releasing hormone (CRH) is produced locally at sites of inflammation and has profound pro-inflammatory effects at an autocrine/paracrine level. We have called this "immune" CRH. Glucocorticoids and somatostatin suppress, and RU 486 markedly augments local secretion of immune CRH at an inflammatory site. Immune CRH was found in the ovary and endometrium, where it may participate in the inflammatory phenomena of ovulation, luteolysis, blastocyst implantation, and menstruation. Patients with rheumatoid arthritis have defective pituitary-adrenal axis responses to inflammatory stimuli and produce excessive amounts of immune CRH in their inflamed joints. Patients with multiple sclerosis have mild hypercortisolism, which is sustained by chronic hypothalamic AVP rather than CRH hypersecretion. The human CRH gene contains estrogen-responsive elements in its promoter region providing an explanation for the sexual dimorphism in the incidence of autoimmune/inflammatory disease. CRH antagonists may be useful in the treatment of autoimmune/inflammatory diseases.

Professional Personnel: (continued)

Others:	C. Bamberger	Guest Researcher	SPE, DEB, NICHD
	M. De Castro	Guest Researcher	SPE, DEB, NICHD
	I. Elenkov	Guest Researcher	SPE, DEB, NICHD
	D. Papanicolaou	Visiting Associate	SPE, DEB, NICHD
	C. Stratakis	Visiting Associate	SPE, DEB, NICHD
	C. Tsigos	Visiting Associate	SPE, DEB, NICHD
	E. Webster	IRTA Fellow	SPE, DEB, NICHD
	K. Zachman	Bio Lab Technician	SPE, DEB, NICHD

Project Description:

We are studying the involvement of interleukin-6 (IL-6) in the interactions between the inflammatory/immune response and the endocrine system and its products. We have shown that IL-6 is one of the most potent stimuli of the hypothalamic-pituitary-adrenal (HPA) axis and arginine-vasopressin (AVP) secretion in man. We are employing recombinant IL-6 as a provocative stimulus of the corticotropin releasing hormone (CRH) neuron in humans with potential alterations in the secretion of this neuropeptide.

Given to rats, the glucocorticoid antagonist RU 486 enhances the inflammatory response and causes enlargement of the thymus and spleen, presumably by antagonizing the immunosuppressive effects of endogenous glucocorticoids exerted at physiologic secretion rates.

One of the potential mechanisms by which RU 486 potentiates inflammation is by stimulation of "immune" CRH production at the level of the inflammatory site. We have shown that the latter is a major pro-inflammatory agent in vivo and that its gene is responsive to estrogen, suggesting that it may play a major role in the sexual dimorphism of the inflammatory/immune response and inflammatory/autoimmune disease. The incidence of the latter is much higher in females than males.

RU 486 allowed the identification of a central nervous system defect in Lewis rats, a strain prone to development of autoimmune arthritis and other inflammatory states. In these animals the glucocorticoid response to stress-mediators is inadequate to restrain the immune system following an insult. This pathophysiologic mechanism is novel and of relevance to human rheumatoid arthritis and other inflammatory/autoimmune diseases. We have demonstrated that patients with rheumatoid arthritis have subnormal responses of the hypothalamic-pituitary-adrenal axis to inflammatory stimuli, which can explain their unrestrained inflammatory response in the joints and other tissues.

Objectives:

The objectives of this project are to increase our understanding of the physiologic interactions between the endocrine and immune systems and of the impact of alterations in these interactions on the development of human disease. The knowledge obtained may assist us with improving the diagnosis and treatment of diseases in which these interactions are disturbed.

Methods Employed:

Methods consist of glucocorticoid and other receptor assays, and in vivo tests of glucocorticoid action (thymus involution, glycogen deposition, growth retardation, suppression of inflammation). Radioimmunoassays and/or ELISA assays for measurement of pertinent hormones or cytokines are also used. We have developed a thin layer and high performance liquid chromatography (HPLC) method and a radioreceptor assay procedure to purify and measure RU 486 in biological fluids. We have also cloned human glucocorticoid receptor mutants/isoforms in an expression vector and employ a co-transfection system in which a glucocorticoid-responsive reporter gene is used to study the functional properties of these mutants/isoforms. We use in situ hybridization and Northern blots to measure expression of glucocorticoid, POMC, AVP and AVP receptor and CRH, CRH receptor and CRH binding protein mRNA in the hypothalamus, pituitary, and other tissues. We use standard immunohistochemistry and/or extraction-HPLC-radioimmunoassay to demonstrate presence and distribution of CRH and other neuropeptides and cytokines in tissues. We also employ a hypothalamic organ culture system for examination of CRH and AVP secretion and a dispersed anterior pituicyte culture system for examination of ACTH secretion. We employ standard nucleic acid extraction and cloning and sequencing techniques. We have developed our own antibodies against CRH and other peptides, as well as against isoforms of the glucocorticoid receptor, for performing assays, immunohistochemistry, Western blots, and/or immunoneutralization studies.

Progress:

Effects of Interleukin-6 in Man

We have performed dose-response studies with recombinant interleukin-6 in humans by administering the cytokine parenterally. Interleukin-6 causes sustained elevations of plasma ACTH, cortisol and arginine-vasopressin, without major side effects, such as hypotension or vascular leak phenomena, as one observed earlier with tumor necrosis factor-alpha (TNF-α) and Interleukin-1. We have demonstrated that exercise causes an intensity-dependent elevation of plasma IL-6 concentration, which is suppressed by pretreatment of the subjects with glucocorticoids. Patients with glucocorticoid deficiency have elevated plasma concentrations of IL-6. We have demonstrated by cross-correlation analysis of plasma IL-6, ACTH and cortisol concentrations in patients with early, untreated rheumatoid arthritis, that IL-6 has a circadian rhythm with a morning surge preceding that of ACTH and cortisol. We found the IL-6 drives ACTH and cortisol in the morning, but is also suppressed by the latter at later times.

Usefulness of the Glucocorticoid Antagonist RU 486

The availability of RU 486 provided the unique opportunity to study two longstanding questions on the effects of glucocorticoids on the immune system and inflammatory response. First, are these effects glucocorticoid receptor-mediated? And second, do endogenous glucocorticoids exert an effect on the immune system at physiologic secretion rates? In an "aseptic inflammation" rat model, glucocorticoids suppress exudate formation, leukocyte diapedesis and prostanoid accumulation. RU 486 was able to antagonize the effect of pharmacologic doses of glucocorticoids, suggesting that they are receptor-mediated. In addition, RU 486 given alone caused enhancement of the inflammatory response, suggesting that endogenous glucocorticoids at physiological secretion rates exert suppressive effects on the inflammatory response in the rat. RU 486 also caused enlargement of the thymus and spleen in these animals. These promising observations suggested that a glucocorticoid antagonist could be used as an immune enhancer in conditions requiring such enhancement (cancer, immunodeficiency).

We recently found that human tissues contain an endogenous non-ligand binding glucocorticoid receptor isoform, which acts a dominant negative inhibitor of the glucocorticoid receptor. This inhibitor may define the sensitivity of the immune system to glucocorticoids. Increased expression might cause glucocorticoid resistance and, hence, a hyperactive immune/inflammatory reaction. Decreased expression might cause glucocorticoid hyposensitivity and hence, immunosuppression. Whether this isoform is involved in the pathogenesis of autoimmune/inflammatory disease is examined under project Z01 HD-00618-14-DEB.

RU 486 was instrumental in identifying a central nervous system defect in rats prone to arthritis in response to antigenic stimulation with streptococcal cell wall polysaccharide (SCW). These rats (Lewis rats) had deficient CRH, ACTH and glucocorticoid responses to stressors, including inflammatory stimuli. "Histocompatible" rats (Fischer rats), which normally do not develop arthritis in response to SCW, do so when treated with RU 486. We defined the CNS defect of Lewis rats in the hypothalamic CRH neuron, which is globally underresponsive to the inflammatory cytokines, and the stimulatory neurotransmitters serotonin, acetylcholine and norepinephrine. We believe the defect is in the 5' regulatory region of the CRH gene or more likely in one of the related transcription factors (see specific section below).

"Tissue" CRF's vs. "Immune" CRH

The presence of non-hypothalamic "tissue" CRFs was suggested by Brodish in the early 70's. The concept was revived by Woloski et al. in 1985. We have employed a specific antibody to prove both the existence of an immunoreactive "tissue" CRH and its importance for the immune/inflammatory reaction in vivo, in the whole animal. This is separate from the various humoral mediators of inflammation, such as the inflammatory cytokines TNF-α, interleukin-1 and interleukin-6, or lipid mediators of inflammation, such as several eicosanoids and platelet activating factor (PAF), which stimulate CRH neuron function and/or pituitary ACTH secretion in vitro and/or in vivo.

A number of recent reports suggested that the interactions between the nervous, endocrine and immune system were more extensive than those mentioned above. These recent studies presented evidence that CRH had direct effects upon leukocytes. For example, CRH directly stimulated production of proopiomelanocortin (POMC)-related peptides and secretion of interleukin -1 and -2, stimulated lymphocyte proliferation, enhanced the proliferative response of leukocytes to lectins and increased the expression of the IL-2 receptor on T lymphocytes. These reports were complemented by the finding of specific CRH binding sites in various subpopulations of leukocytes and, finally, by presence of CRH immunoreactivity and CRH mRNA by in situ hybridization and Northern blotting, in resting subpopulations of human leukocytes. All these data suggested strongly that CRH might have local direct effects upon the immune/inflammatory system. We attempted to identify the potential biological role of these effects by first examining the ability of systemic CRH immunoneutralization to influence the size of a quantifiable inflammatory response. As such, we employed the aseptic subcutaneous reaction to carrageenin.

We studied male Sprague-Dawley rats that were injected intraperitoneally with neutralizing rabbit anti-CRH antisera one hour before subcutaneous injection of carrageenin. Both exudate volume and cell concentrations were suppressed by approximately 50-60%, whereas no such suppression was observed in control rats pretreated with normal saline, normal rabbit sera or anti-TSH rabbit antisera. The specific suppression of the inflammatory response observed after CRH immunoneutralization was clearly opposite to what could be expected from abrogation of hypothalamic-hypophyseal portal CRH. The latter should have resulted in relative hypoglucocorticoidism and enhancement of the inflammatory response, as is seen in animals pretreated with a glucocorticoid receptor antagonist. These findings are instead compatible with the view that local effects of CRH are proinflammatory. This concept was complemented by two different sets of data. First, parallel experiments with TNF-α neutralizing antisera also showed marked suppression of inflammation, approximately of the same magnitude as that observed with anti-CRH antisera. TNF-α is a major auto/paracrine proinflammatory agent, which directly, or via IL-1 and/or IL-6, activates the inflammatory response. No additive effect was observed between anti-CRH and anti-TNF, a finding compatible with common or shared mechanisms of inflammation suppression between these two antisera. Second, direct local administration of anti-CRH antisera into the air pouch also decreased the inflammatory reaction.

Since the above findings suggested that CRH might participate in the inflammatory process in vivo as a local stimulatory agent, we examined the site of inflammation for evidence of CRH production. Several approaches were taken. First, significantly high amounts of immunoreactive CRH were intracellularly detected in the inflamed tissues by specific immunohistochemistry, using an affinity-purified anti-CRH antibody. Second, direct measurement of CRH immunoreactive content of extracted inflammatory tissue demonstrated a high quantity of CRH immunoreactivity at the range of 400pg/g of wet tissue. High CRH concentrations have also been demonstrated in the rat hypothalamus and human placenta. Sizing of the CRH-immunoreactive molecule by HPLC revealed a fraction eluting at the location of rat/human CRH 1-41, suggesting that CRH acting in inflammatory sites, designated "immune CRH', is similar in chromatographic mobility to that produced by the hypothalamus and the human placenta. These findings, taken together, strongly suggest that there is a local production of a CRH-like molecule by inflammatory tissues, whose immunoneutralization results in decrement of inflammation. The tissue concentrations of this molecule are of sufficiently high concentrations to produce biological responses, including POMC-derived peptide secretion and regulation of immune functions. We have demonstrated that exogenous glucocorticoid or somatostatin suppress immune CRH secretion in a dose-dependent fashion, in parallel to the suppression of the inflammatory/immune response.

To examine whether immunoreactive CRH produced locally, at the inflammatory site, might have distant, endocrine effects, we measured plasma concentrations of IR-CRH in carrageenin- and placebo-treated animals through the 7h ensuing carrageenin or placebo administration. The concentrations of plasma CRH were quite low, at the range of 8-10pg/ml (~10^{-12}M) and similar in the two groups of animals throughout the experiment. These levels are markedly lower than those of the rat hypophyseal portal blood and not expected to be bioactive in vivo or in vitro. Based on these data we suggest that immune CRH is an auto/paracrine, rather than endocrine, hormone and that rapid metabolism prevents it from reaching biologically significant circulating concentrations. In this sense, it is similar to other autacoids with known major proinflammatory or anti-inflammatory activity, of which platelet activating factor (PAF) and prostaglandin E_2 are examples. Other paradigms of peripheral CRH secretion with primarily auto/paracrine function is its production by chromaffin cells of the sympathetic ganglia and adrenal medulla and by Leydig cells of the testis. In the former it appears to potentiate catecholamine secretion, while in the latter it seems to be involved in an apparent autocrine inhibitory feedback on testosterone production.

The data from our study suggests that CRH plays a dual, antithetical-albeit counterbalanced role in the regulation of the inflammatory/immune response. Indirectly, hypothalamic CRH suppresses this response by activating glucocorticoid secretion and causing increased sympathetic outflow, two phenomena leading to immunosuppression. Immune CRH, on the other hand, has potent proinflammatory actions which can be unmasked by use of neutralizing CRH antisera and, perhaps, peripheral antagonists of this peptide. Does immune CRH have any peripheral roles in addition to its proinflammatory ones? For instance, it may be involved in antinociception by stimulating a known peripheral analgesic, β-endorphin, or by exerting its own direct antinociceptive effects. The latter would be analogous to its recently described antinociceptic effects in the central nervous system. How are the effects of immune CRH exerted on the immune system at a molecular level? Its major second messenger in the pituitary corticotroph is cyclic AMP(4) which has been primarily shown to suppress immune functions when elevated. Would this suggest that immune CRH acts through a different receptor or second messenger system? Many questions have been generated from this study and clinical applications of immune CRH agonists or antagonists as potentiators or inhibitors of the inflammatory/immune response can be envisioned.

Secretion of immune CRH in inflammatory sites appears to be a generalized phenomenon. We extended our observations to several experimental and human states, including retinol-binding protein-induced uveitis in rats and mice, and human rheumatoid arthritis and Hashimoto thyroiditis. Immunoneutralization of CRH and TNF-α ameliorated uveitis in mice only when given in the early phase of the disease, suggesting that both peptides play a major role in the initiation and early propagation of inflammation. Glucocorticoids and somatostatin suppresed immune CRH secretion, in accordance with their antiinflammatory effects. Glucocorticoids, in fact, stimulated somatostatin secretion within the inflammatory sites and antisomatostatin antibodies inhibited in part the antiinflammatory actions of glucocorticoids, suggesting that somatostatin may play the role of an intermediary auto-paracrine messenger.

Immune CRH in Reproductive Tissues

Since both ovulation and luteolysis and blastocyst implantation and menstruation represent aseptic inflammatory phenomena, we considered the ovary and endometrium as potential sites of CRH secretion and action. Indeed we have demonstrated secretion of CRH in both the rat and human ovary as well as the presence of specific CRH receptors in these gonads. The theca and stroma as well as the corpora lutea contained both. We are currently studying the role(s) of CRH in the ovary along with its second messenger system in ovarian cells. We found decreased CRH expression in the theca and stroma of human polycystic ovaries. We also found that CRH appears to inhibit steroidogenesis by cultured luteinized granulosa cells. This suggests that androgen hypersecretion by polycystic ovaries may be related to decreased inhibition by CRH. Recently, we also demonstrated CRH in the glandular epithelium of the human endometrium at both phases of the cycle, and within the endometrial stromal cells and local macrophages in the luteal phase.

A Central Nervous System Defect Linked to Autoimmune/Inflammatory Disease

We have demonstrated that the propensity of Lewis rats to develop severe inflammatory arthritis in response to streptococcal cell wall polysaccharide (SCWP) is related to their inability to mount an adequate counter-regulatory activation of their HPA axis during the inflammatory response. "Histocompatible" Fischer rats, which normally do not develop arthritis in response to SCWP, do so, when treated with the glucocorticoid antagonist RU 486. The diminished HPA axis response in Lewis rats appears to result from a generalized defect in the synthesis and secretion of hypothalamic CRH in response to different types of physiologic and pharmacologic stimuli. We believe that a defect in the CRH neuron of Lewis rats, which makes it globally under-responsive to IL-1, serotonin, acetylcholine norepinephrine and other physiologic regulators, is responsible for the hyposecretion of glucocorticoids during the immune/inflammatory response. Interestingly, the Sprague-Dawley rat, from which the first histocompatible Lewis and Fischer rats were bred, has an intermediate position between the two other strains in both HPA axis activity and propensity to develop autoimmune inflammatory disease in response to SCWP. We have studied in detail the basal and stress-induced pattern of ACTH and corticosterone secretion in the Lewis and Fischer rats. A blunted circadian rhythm, with lowered time-integrated ACTH and corticosterone secretion, and hyporesponsiveness to any kind of stress (inflammatory, immobilization, and other) characterizes the Lewis rat. We extended the original findings with SCWP to another form of inflammation, the carrageenin model - with similar differences between the strains. Interestingly, although the hypothalamic CRH response was decreased in Lewis rats compared to Fischer rats, copious amounts of IR-CRH were found in the joints and carrageenin inflammation sites of the former. This suggests that both lack of glucocorticoid-induced suppression and excess of local CRH secretion in the Lewis rat may be responsible for the excessive inflammatory response observed in this strain, and that the regulation of CRH synthesis/secretion is different between the CNS and the periphery. The latter could be explained by different tissue-specific regulation or be deficient local glucocorticoid-mediated suppression of immune CRH synthesis/secretion. Interestingly, Lewis rats had elevated plasma levels of arginine vasopressin (AVP) compared to Fischer rats. Immunoneutralization of AVP ameliorated carrageenin-induced inflammation, suggesting that AVP participates in inflammation as a proinflammatory agonist. The Lewis and Fischer rats have another fascinating property, when compared to the maternal Sprague-Dawley rat strain. The former has behaviors characteristic of low central nervous system (CNS) CRH, whereas the latter has behaviors typical of high CNS CRH. These strains represent, thus, interesting models of stress system hypo- and hyperfunction, reminiscent of the data described in atypical and melancholic depression, respectively.

The major question comes up whether the above findings bear any relevance to human pathophysiology. We have demonstrated presence of high levels of IR-CRH with the chromatographic mobility of hCRH 1-41 on HPLC in synovial fluid and histologic specimens from joints of patients with rheumatoid arthritis. Severe behavioral problems in such patients have been noted, reminiscent of atypical depression, and the question as to whether a subgroup of human patients with rheumatoid arthritis or other autoimmune inflammatory diseases have inadequate central "endocrine" counter-regulatory responses upon the immune/inflammatory response, combined with behavioral traits related to changes in the regulation of the HPA axis, is actively being pursued. We have evidence that patients with rheumatoid arthritis are indeed hyporesponsive to inflammatory stimuli, having thus pathophysiologic mechanisms similar to those observed in the Lewis rat. We are planning to study this further by employing recombinant interleukin-6 (IL-6) as a test stimulus in patients with autoimmune diseases or vulnerability to such diseases.

We studied the HPA axis of patients with fibromyalgia and multiple sclerosis. The former had evidence of hypofunction of this axis, similarly to patients with chronic fatigue syndrome. The latter had mild chronic hypercortisolism, which however was sustained by high hypothalamic AVP rather than CRH secretion. We also studied the HPA axis of postpartum women, given that this period is characterized by increased incidence of autoimmune/inflammatory diseases. We found transiently suppressed hypothalamic CRH secretion in these patients, which would explain their increased vulnerability to such diseases (please see project Z01 HD-00618-14-DEB).

Significance to Biomedical Research and the Program of the Institute:

Interleukin-6 is a very potent and relatively innocuous stimulus of the HPA axis in man. We have shown that it carries promise as a diagnostic tool and as a probe to study adrenal physiology. As a challenger of the hypothalamic-pituitary-adrenal axis it holds promise in the study of disorders of the axis: Cushing's syndrome, adrenal insufficiency, psychiatric hypercortisolism and hypocortisolism and vulnerability to psychiatric and autoimmune disease. As an experimental tool, RU 486 provides an excellent means to study stress physiology and the role of glucocorticoids upon the immune system and the inflammatory response. Recently, RU 486 gave us new insight on the pathogenesis of arthritis and/or other inflammatory/autoimmune diseases. In these studies, CRH was found to be a major local cytokine, influencing positively the inflammatory response. The CRH gene was found to be responsive to estrogen, suggesting that the female-male differences in the inflammatory/immune response and the incidence of inflammatory/autoimmune disease might be explained by sexual dimorphism in the regulation of this gene. CRH was found to participate in ovarian and endometrial functions, probably promoting physiologic aseptic inflammatory processes. Preliminary studies suggest that it may also play a role in blastocyst implantation.

Proposed Course:

1) We have used interleukin-6 as a new provocative test of hypothalamic-pituitary-adrenal function. We are now completing studies with psychiatric hypercortisolism (melancholic depression, pseudoCushing) and hypocortisolism (atypical depression, chronic fatigue-fibromyalgia, postpartum blues/depression) and autoimmune/inflammatory disease (rheumatoid arthritis, Hashimoto thyroiditis), conditions in which the pituitary ACTH reserve or the glucocorticoid negative feedback may be significantly altered. Of special interest would be the detection of individuals premorbidly, to define who is vulnerable to develop a disease characterized by a hyperresponsive (melancholic depression, panic anxiety) or hyporesponsive (atypical depression, chronic fatigue/fibromyalgia, postpartum blues/depression, autoimmune/inflammatory diseases) HPA axis.

2) We are examining the effects of stress hormones, including glucocorticoids and the catecholamines, on the ability of human monocytes/macrophages to secrete interleukin 12 and 10, the key regulators of cellular and humoral immunity, respectively.

3) We are now defining the anatomic locus and the molecular mechanisms of the defective HPA axis response of Lewis rats. It appears that there is generalized deficiency of CRH neuron responsiveness to various stimuli. We plan to clone the 5' regulatory region of the CRH gene in these animals, to sequence it and to examine its functional activity in the presence of Lewis and Fischer rat hypothalamic nuclear extracts.

4) It is of utmost importance to define the actual cellular target(s) of immune CRH, to demonstrate the presence of CRH receptors in these tissues, to identify the type of receptors (CRH-R1, -R2α or -R2β) present and to show whether the recently characterized CRH-responsive element-binding protein is present in such target cells. We plan to employ newly developed CRH antagonists to explore the therapeutic potential of these compounds in inflammatory states as topical antiinflammatory drugs or as anti-edema/anti-vascular permeability agents.

5) We plan to examine the potential functional significance of immune CRH in reproductive tissues, using newly developed potent CRH antagonists. We have a particular interest in the phenomenon of blastocyst implantation, an aseptic inflammation-like process.

Protocols:

Human

82-CH-45	Chrousos	Dose-Response Relationships for Single Doses of Corticotropin-Releasing Hormone (active) This blanket protocol enables us to administer ovine corticotropin-releasing hormone (CRH) as a provocative test of the hypothalamic-pituitary-adrenal (HPA) axis in men, women, and children with potential alterations of this axis. The peptide continues to be an investigational drug (IND# 19802) and all data concerning its

administration are captured and reported annually to the FDA. During the past year oCRH was administered under this and other approved protocols.

88-CH-120	Chrousos	The Hypothalamic-Pituitary-Adrenal Axis in Pregnancy, the Postpartum Period and in Postpartum Depression Syndromes (precis and brief analysis included in project Z01 HD-00618-14-DEB)
94-CH-134	Papanicolaou	Dose-Response Relationships for Single Doses of Recombinant Interleukin-6 in Normal Volunteers and Patients with Disorders of the Hypothalamic-Pituitary-Adrenal (HPA) Axis This protocol concerns the administration of recombinant interleukin-6 to normal volunteers and patients with disorders of the HPA axis. Interleukin-6 is an investigational drug (IND# 5419) with which we have already obtained some experience from a phase 1 study of cancer patients done by NCI. A dose-response curve in normal volunteers has been completed and a dose has been selected to be administered to the various patient groups listed in the protocol. The hypothesis of the protocol is that as a hypothalamic stimulus of CRH, acute administration of interleukin-6 will distinguish biochemically not only patients with a clearly disordered HPA axis, i.e. Cushing's vs. pseudoCushings's vs. melancholic depression vs. chronic active alcoholism or melancholic vs. atypical depression, but also premorbid states of vulnerability, i.e. individuals prone to melancholic depression or anxiety disorders or individuals prone to atypical depression, chronic fatigue/fibromyalgia, postpartum blues or depression, alcoholism or autoimmune disorders.

Animal Protocols:

89-036 rat	Chrousos	Regulation of Hypothalamic CRH and Pituitary ACTH Release (active)
91-036 rat	Chrousos	Dose-Response Studies Between Dexamethasone and Suppression of Thymic and Adrenal Weights in Fischer and Lewis Rats (completed)
92-032 rat	Webster	Reproductive Tissue Inflammation-like Processes and Local CRH Production (active)
95-014 rat	Chrousos	Role of Corticotropin Releasing Hormone in Implantation (active)
96-007 rat	Chrousos	CRH Receptor in Inflammation: Localization and Characterization

Publications:

Bamberger CM, Chrousos GP. The glucocorticoid receptor and RU 486 in man. Ann NY Acad Sci 1995;761:296-311.

Chrousos GP. Cytokine therapy in septic shock. (Letter to the Editor). N Engl J Med 1995;333:942-3.

Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med 1995;332:1351-62.

Chrousos GP, McCarty R, Cizza G, Pacak K, Sternberg E, Gold PW, Kvetananski R. (eds.) Stress: mechanisms and clinical implications. Ann NY Acad Sci 1995;771: New York. (775 pages, ISBN 0-89766-943-6, cloth; 0-89766-944-4, paper)

Crofford LJ, Sano H, Karalis K, Webster EA, Friedman TC, Chrousos GP, Wilder RL Local expression of corticotropin-releasing hormone in inflammatory arthritis. Ann NY Acad Sci 1995;771:459-71.

Elenkov IJ, Papanicolaou DA, Wilder RL, Chrousos GP. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. Proc Assn Am Phys

Magiakou MA, Chrousos GP. Glucocorticoid therapy and withdrawal. In: RC Bone. Current Practice of Medicine. Current Medicine, Inc., Philadelphia, IV 1996;6.1-.6.

Masi A, Chrousos GP. The hypothalamic-pituitary-adrenal-glucocorticoid axis in rheumatoid arthritis. J Rheumatol 1996;23:577-81.

Mastorakos G, Bouzas E, Caspi R, Friedman T, Chrousos GP. Immune corticotropin releasing hormone (CRH) is present in the eyes of and promotes experimental autoimmune uveoretinitis (EAU) in rodents. Endocrinology 1995;136:4650-8.

Mastorakos G, Magiakou MA, Chrousos GP. Effects of the immune/inflammatory reaction on the hypothalamic-pituitary-adrenal axis. Ann NY Acad Sci 1995;771:438-48.

Papanicolaou DA, Tsigos C, Oldfield EH, Chrousos GP. Acute glucocorticoid deficiency is associated with plasma elevation of interleukin-6: does the latter participate in the symptomatology of the steroid withdrawal syndrome and adrenal insufficiency? J Clin Endocrinol Metab 1996;81:2303-6.

Papanicolaou DA, Petrides JS, Tsigos C, Bina S, Kalogeras KT, Wilder R, Gold PW, Deuster PA, Chrousos GP. Exercise stimulates interleukin-6 secretion: inhibition by glucocorticoids and correlation with catecholamines. Am J Physiol

Reincke M, Lehmann R, Karl M, Magiakou MA, Chrousos GP, Allolio B. Severe illness. Neuroendocrinology. Ann NY Acad Sci 1995;771:556-69.

Theoharides TC, Spanos C, Pang Z, Alferes L, Ligris K, Letourneau R, Rozniecki JJ, Webster E, Chrousos GP. Stress-induced intracranial mast cell degranulation. A corticotropin-releasing hormone-mediated effect. Endocrinology 1995;136:5745-50.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00618-15 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Physiology and Pathophysiology of the Hypothalamic–Pituitary–Adrenal and Gonadal Axes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G.P. Chrousos	Head	SPE, DEB, NICHD
Others:	See attached list.		

COOPERATING UNITS *(if any)*

See attached list.

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Pediatric Endocrinology

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
8	6.6	1.4

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In this project we seek to advance the understanding of the physiology and pathophysiology of the hypothalamic–pituitary–adrenal (HPA) and –gonadal (HPG) axes. The role of stress–related hormones in normal and disease states is being examined, and clinical applications for these hormones are sought. The discovery of the structure of corticotropin releasing hormone (CRH) and the development of sensitive assays for measuring HPA– and HPG–related hormones and their receptors have led to rapid progress in this field. We recently demonstrated that the postpartum blues/depression syndrome is characterized by transient profound hypoactivity of the stress system, which explains not only the mood disorder but also the propensity of these patients to develop autoimmune diseases. We also found the hCRH gene 5' regulatory region has 2 active promoters and responds positively to estrogens, providing a potential explanation for the sexual dimorphism of psychiatric diseases characterized by aberrations in CRH secretion. Glucocorticoid resistance is an autosomal recessive or dominant disease associated with abnormalities of the glucocorticoid receptor. We have elucidated the molecular pathophysiology of this syndrome by defining mutations and/or deletion of the glucocorticoid receptor gene leading to abnormal or decreased receptors in the tissues of patients. The mineralocorticoid receptor and the subunits of the amiloride–sensitive sodium channel were studied in sporadic cases of pseudohypoaldosteronism or mineralocorticoid resistance and the defect was localized it in the β and γ subunits of this channel. The interactions of the classic glucocorticoid receptor (GRα) and its nonligand binding natural homolog glucocorticoid receptor β (GRβ) with each other and with the heat–shock proteins and glucocorticoid–responsive elements (GREs) of the DNA are studied, as the well as the importance of GRβ in human physiology and pathophysiology. We have found an abnormal expression of the β vs. the α isoform of the glucocorticoid receptor in patients with glucocorticoid resistant asthma. We have elucidated the molecular pathophysiology of hereditary ACTH resistance, an autosomal recessive disorder characterized by isolated glucocorticoid deficiency, by defining abnormalities of the ACTH receptor gene. We have elucidated the molecular pathophysiology of testicular and ovarian resistance to luteinizing hormone (LH) by defining abnormalities of the LH receptor gene. We have localized the gene for Carney Complex, a multiple neoplasia/lentiginosis syndrome, on chromosome 2p16, and have characterized it as a protooncogene. We have defined the molecular defect in the excessive aromatase syndrome, a novel promoter aberrantly and ectopically expressed.

Professional Personnel: (continued)

Others:	K. Arai	Visiting Fellow	SPE, DEB, NICHD
	C. Bamberger	Guest Researcher	SPE, DEB, NICHD
	G. Cizza	Visiting Associate	SPE, DEB, NICHD
	M. De Castro	Guest Researcher	SPE, DEB, NICHD
	T. Kino	Visiting Fellow	SPE, DEB, NICHD
	M. Karl	IRTA Fellow	SPE, DEB, NICHD
	A.C. Latronico	Guest Researcher	SPE, DEB, NICHD
	D. Papanicolaou	Visiting Associate	SPE, DEB, NICHD
	M. Ramayya	Clinical Associate	SPE, DEB, NICHD
	C. Stratakis	Visiting Associate	SPE, DEB, NICHD
	S. Taymans	Predoctoral IRTA	SPE, DEB, NICHD
	C. Tsigos	Visiting Associate	SPE, DEB, NICHD
	E. Webster	IRTA Fellow	SPE, DEB, NICHD
	K. Zachman	Bio Lab Technician	SPE, DEB, NICHD
	A. Vottero	Special Volunteer	SPE, DEB, NICHD
	S. Yanovski	Special Volunteer	SPE, DEB, NICHD

Cooperating Units:

P.W. Gold, Clinical Neuroendocrinology Branch, NIMH; E. Oldfield, Surgical Neurology Branch, NINDS; P. Deuster, Human Performance Laboratory, Dept of Military Medicine, USUHS; A. Magiakou, G. Mastorakos, Athens University Medical School, Greece; M. Reincke, University of Wurtzburg, Germany; A. Vgontzas, Pennsylvania State University, Hershey, PA; L. Dorn, University of Pennsylvania, Pittsburg, PA; A.J. Carney, Mayo Clinic, Rochester, MA; Mark Hughes, Human Genome Institute, NIH; D.Y. Leung, University of Colorado Health Sciences Center, Denver, CO.

Project Description

Objectives:

The term stress encompasses the sum of body reactions that take place during the influence of stimuli (stressors) threatening to alter homeostasis. The changes occurring are both behavioral and physical. Stress is associated with activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic system. It appears that these two systems interact at various levels, from the CNS to the peripheral target tissue, and when activated have profound effects on other systems, including those subserving reproduction, growth and immunity. The structure of CRH, the principal hypothalamic hormone regulating pituitary-adrenal function, was discovered in 1981 after over 20 years of investigation. This discovery made it possible to begin to explore the role of this hormone in stress and in human health and disease. The general objectives of this project have been to understand the physiologic and pathophysiologic mechanisms of stress in experimental animals and man and to study the function of the hypothalamic-pituitary-adrenal axis in stress, health and disease. For clarity, we have divided the "Progress", "Significance to Biomedical Research and the Program of the Institute" and "Proposed Course" sections of the annual report into three broad sections: a) Clinical Applications and Implications of CRH, b) Regulation of the Hypothalamic-Pituitary-Adrenal Axis In Vivo and In Vitro, c) Roles, Actions and Pathophysiology of HPA and Hypothalamic-Pituitary-Gonadal (HPG) Axes Hormones, and d) Mechanisms of Adrenocortical Tumorigenesis.

Methods Employed:

1) Metabolic clearance studies - Methods consist of (1) radioactive labeling and chromatographic purification of a hormone or (2) measurement of that hormone by radioimmunoassay, immunoradiometric assay or enzyme-linked assay and estimation of its metabolic clearance rate by the pulse injection or continuous infusion methods.

2) Radioimmunoassay (RIA)/Immunohistochemistry - RIAs have been developed to measure ovine and human CRH, ACTH and POMC-derived peptides such as beta-endorphin and alpha-MSH, as well as other peptides. Also, RIAs have been developed for vasopressin, dynorphin A, tumor necrosis factor-α (TNF-α), δ-sleep-inducing peptide and neuropeptide Y. Affinity purification of antibody and immunohistochemistry procedures for several of the above peptides have been set up.

3) Peptide hormone receptor assays have been used to examine the presence and characteristics of these receptors in various tissues or cells.

4) An ACTH bioassay in which rat adrenal corticosterone secretion is examined as an end-point.

5) A pituitary culture system is being used to examine the CRH activity of various extrahypothalamic substances with CRF bioactivity but not immunoreactivity.

6) A hypothalamic organ culture system in which the secretion of CRH from rat hypothalami is examined in vitro. This allows us to directly examine the regulation of CRH neuron function by neurotransmitters, neuromodulators, and feedback systems.

7) An organ perfusion system of human or rat full thickness placenta fragments has been established in which we study the secretion of CRH and POMC-derived peptides. The regulation of placental secretion of these peptides is studied.

8) POMC, CRH, AVP mRNA assays by Northern blotting, RNAse protection, and in situ hybridization are used to determine the site of POMC, CRH, and AVP gene expression as well as their regulation. Similar methods have been established for the CRH and AVP receptors and the CRH-binding protein.

9) Glucocorticoid receptor assays - both binding assays, protein assays by Western blotting and assays of receptor mRNA by Northern blotting or in situ hybridization for both types of the glucocorticoid receptor, glucocorticoid and mineralocorticoid, are performed in our laboratory. The regulation of the glucocorticoid receptor gene expression, especially its alternative splicing to the classic glucocorticoid receptor and its natural nonligand-binding isoform ß, is examined. The molecular pathophysiology of glucocorticoid and mineralocorticoid resistance in man and nonhuman New World primates is examined.

10) Standard cloning and sequencing procedures. These are applied in the cloning of the glucocorticoid, mineralocorticoid, ACTH and LH receptor in families or subjects with cortisol , aldosterone, ACTH and LH resistance, and in the cloning of other genes of choice. Co-transfection assays and use of reporter plasmids expressing chloramphenicol acetyl transferase (CAT) or luciferase coupled to appropriate promoters have been developed to assess the functional activity of several gene products.

11) Standard genetic linkage analyses for chromosomal localization of hereditary endocrine disorders, such as Carney complex and the Triple A syndrome.

Progress:

A. Clinical Applications and Implications of CRH

From testing normal subjects it has become evident that age plays no major role in the responsiveness of plasma ACTH and cortisol to CRH in man. Children above five to old men in their nineties have virtually indistinguishable responses. There is a small difference in the responsiveness of the axis between men and women characterized by exaggerated ACTH responses and slight prolongation of the cortisol responses in the latter. There are no changes in the responsiveness of the HPA axis during the different phases of the menstrual cycle in normal women.

CRH causes elevations of plasma ACTH and cortisol in patients with pituitary Cushing's disease, whereas it does not in patients with the ectopic ACTH syndrome. The differential diagnostic accuracy of the CRH test in this condition exceeds 85 percent. Patients with Cushing's syndrome of adrenal etiology have low or undetectable levels of plasma ACTH, that fail to respond to exogenous CRH. Thus, the CRH stimulation test is a useful out-patient, brief, safe and easy diagnostic tool in the differential diagnosis of Cushing's syndrome, as it distinguishes pituitary from adrenal or ectopic causes, each requiring a different therapeutic approach. A prospective study of our patient series suggests that the oCRH test is equal, or superior, in its predictive value than the standard tests which are in-patient, long, and cumbersome.

Pituitary Cushing's disease is usually due to small ACTH-secreting adenomas. Cure is achieved by transsphenoidal adenomectomy. We have improved the cure rate by introducing a new sampling method in association with oCRH administration. We are sampling the veins draining the pituitary gland (petrosal sinuses) simultaneously for measurement of ACTH both basally and after CRH. In the overwhelming majority of patients an ACTH concentration gradient is found between one or both petrosal sinuses and a peripheral vein. Thus, the surgeon searches for a pituitary adenoma. Continuous ovine CRH administration as early as a week after successful, curative surgery stimulates ACTH and cortisol levels into the normal range, suggesting that the profound adrenal insufficiency normally observed in cured patients is of hypothalamic or suprahypothalamic origin.

In adrenal insufficiency, testing with CRH helps differentiate between hypothalamic and pituitary causes, since in the former there are ACTH responses to CRH, whereas in the latter there are not. It is interesting that hCRH, which we have shown to be short-acting and reproducing the spontaneous secretory episodes of ACTH and cortisol, restores, when given in a pulsatile fashion, the normal pattern of ACTH and cortisol in patients with hypothalamic adrenal insufficiency. Glucocorticoid therapy administered on alternate days is associated with a normal ACTH and cortisol response to CRH on the day off. Interestingly, adrenal androgen responses are suppressed suggesting that alternate therapy may be a good therapeutic option for growing children with hyperandrogenism. Patients with the late-onset form of congenital adrenal hyperplasia had an extensive evaluation of the HPA axis. Interestingly, it appeared that the equilibrium between ACTH and cortisol in these patients as a group was totally normal. We considered this an inherent long-standing adaptation of the HPA axis in these patients.

Patients with psychiatric disorders associated with hypercortisolism (depression, panic anxiety, anorexia nervosa) have decreased plasma ACTH responses to exogenous CRH. The blunting of the response is inversely proportional to the degree of hypercortisolism. Thus, patients with pseudo-Cushing's states could potentially be differentiated from patients with Cushing's disease who respond differently to exogenous CRH. The diagnostic accuracy of the test is approximately 75% in distinguishing these two conditions. Pretreatment of the patients with dexamethasone improves significantly the distinction between the two conditions.

Recently, the role of CRH has been expanded from merely that of an ACTH secretagogue to that of a neuromodulator of broader significance. Intracerebroventricular administration of CRH in rats causes physiological (HPA axis activation, sympathetic activation, hypogonadism) and behavioral (aggression, irritability, anorexia, loss of libido) changes that are normally observed in stress. These findings have suggested a pathophysiologic role of CRH in psychiatric conditions characterized by chronic hypercortisolism. If this hypothesis is correct, treatments directed towards preventing endogenous CRH hypersecretion or CRH antagonists may be developed for these conditions.

We compared the diagnostic utility of hCRH to that of oCRH in Cushing's syndrome and psychiatric hypercortisolism. The ovine CRH analog is clearly superior to the human one for diagnostic testing. However, the hCRH analog will remain useful in studies requiring restoration of the physiologic pulsatile pattern of ACTH and cortisol.

We have immunized rabbits with CRH and have produced antisera against both ovine and human CRH. We have used these antisera to produce immunoneutralization in vivo or to develop radioimmunoassays for these peptides. These radioimmunoassays are sensitive to the pg/ml level and have been extensively used to estimate the metabolic clearance rates of these peptides in normal men and patients with adrenal insufficiency, Cushing's syndrome, depression, and anorexia nervosa. Strong stress stimuli, such as surgery, exercise, or insulin-induced hypoglycemia, fail to cause major elevations of plasma CRH immunoreactivity. CRH immunoreactivity can be readily measured in the cerebrospinal fluid (CSF). Its concentration is elevated in depression and anorexia nervosa and decreased in Cushing's syndrome and Alzheimer's disease, and atypical depression. Interestingly, ACTH immunoreactive concentrations in the CSF follow the direction of CRH in the above conditions, suggesting that arcuate nucleus ACTH rather than pituitary ACTH is detected in the CSF.

We examined patients with atypical, seasonal depression. These patients develop dysthymia in the winter months, gain weight and are complaining of fatigue and somnolence. In these patients ovine CRH testing in the summer was totally normal, whereas in the winter it was indicative of secondary hypocortisolism. Interestingly, light therapy normalized the CRH response. We obtained similar data suggesting HPA axis hypofunction in patients with the chronic fatigue syndrome, a condition characterized primarily by fatigue, as well as in patients with fibromyalgia. These findings suggest that a spectrum of diseases exist, in which the pathophysiology is characterized by hypofunction of the central stress system. These syndromes contrast to melancholic depression, panic anxiety and anorexia nervosa, in which we have hyperactivity of the CRH system.

A state-independent abnormally functioning stress system (hyper- or hypo-functioning) might be either a result of different heredity or because an environmental stimulus was exerted at some critical period of life, usually during development (antenatally, infancy, childhood). A state-dependent abnormally functioning stress system, on the other hand, should revert completely to normal once the organism returns to a normal nonpathologic state. Unipolar melancholic depression or atypical depression have alterations of the HPA axis on opposite ends of a spectrum, which could be hereditary and/or constitutional (i.e. a trait) or state-dependent. Evidence from experimental animals and humans accumulates, which indicates that the former is most likely the case. We examined several groups of patients and normals with the purpose of understanding better the relation between personality, stress, depression, and the HPA axis. These were sexually abused girls with dysthymia several years after the abuse took place, children of one or two parents with depression and adolescents with depression, as well as appropriate controls for each of the groups. All of these patients had standardized psychological testing, an oCRH test and measurement of 24h urinary free cortisol excretion. Sexually abused girls had a blunted ACTH response, a normal cortisol response and normal urinary free cortisol excretion. Our interpretation was that these patients had constitutional hypersecretion of CRH but normal negative feedback controls that allowed normalization of free cortisol. Children of one or two parents with depression had normal oCRH testing and urinary cortisol excretion. Adolescents with depression also had normal oCRH testing and urinary cortisol excretion. In the latter two groups we have the following potential explanations: (1) they have normal CRH secretion and the locus of depression is outside the stress system; (2) they are a mixed group of low and high CRH secretors and the averaging is normal; we saw no bimodal distribution; (3) they are low secretors primarily, and our means of examination failed to detect their abnormality; indeed, the age of onset for atypical depression is adolescence and for melancholic depression the third decade of life.

We have found that African Americans have higher plasma ACTH levels than Caucasians in response to CRH. Further study demonstrated that this is due to different metabolism of ACTH 1-39 in blacks vs. whites, with identical ACTH bioactivity, however, in both races.

We have cloned and sequenced the 5' regulatory region of the human CRH gene and identified recognizable signal sequences. The functions of some of these sequences were studied. This gene has 2 different promoters with potentially different regulatory sensitivities, and, in addition, 4 half perfect estrogen-responsive elements that respond positively to estradiol in an estrogen receptor-mediated fashion. The latter suggests that the sexual dimorphism of psychiatric diseases characterized by altered CRH secretion in humans, such as depression and anorexia nervosa, might be explained by the differences in the regulation of this gene.

We hypothesized that marmosets of the species Callithrix jacchus might represent a potentially interesting model for human depression. These New World primates maintain a monogamous breeding system with the males and older siblings participating in the rearing of infants. Subordinate males or females or offspring in the family are reproductively suppressed. Disruption of societal bonds, such as separation of the infant from its parents leads to significant behavioral and physical changes. These include in adults, withdrawn behavior, decreased feeding, loss of weight, and death ("wasting syndrome") and in infants delay or complete cessation of growth and abnormal behaviors. We have documented profound changes in hormones of the HPA axis with degree of subordination, and the wasting syndrome and have shown defective growth of infants abused by immature parents. The growth retarded infants catch up length-wise and improve behaviorally at later stages of their development.

B. Regulation of the Hypothalamic-pituitary-adrenal (HPA) Axis In Vivo and In Vitro.

It has become clear from our studies that opioids are involved in the regulation of the human hypothalamic-pituitary-adrenal axis. Morphine both suppresses the CRH-secreting neuron and/or stimulates some corticotroph suppressing factor. The overall effect of opioids is to suppress the hypothalamic-pituitary-adrenal axis. It has become known from other laboratories that catecholamines, vasopressin and oxytocin stimulate the corticotroph in vitro. Vasopressin also shows synergy with CRH in vivo when the peptides are administered together.

The possibility of peripheral extrahypothalamic CRFs or CRH CRFs participating in the regulation of the HPA axis and playing, in addition, other roles in homeostasis during stress, is a lead that we have followed. CRH at pharmacologic doses causes marked peripheral vasodilation in primates with a concomitant decrease in blood pressure. The major vessels involved were the mesenteric artery and to a lesser extent the iliac arteries. From a survey of peripheral tissues for CRH receptors we have found such receptors in the adrenal medulla and sympathetic ganglia. These receptors are coupled to cyclic AMP and to catecholamine and enkephalin secretion. Receptors on blood vessels or perivascular mast cells may also be present. We are actively searching for peripheral extrahypothalamic (tissue) corticotropin-releasing or CRH releasing-factors. These factors appear to have different immunoreactivity from that of hypothalamic CRH. Immune system products, such as interleukins 1 and 6, thymosin and TNF-α, or tissue growth factors, such as epidermal growth factor, are potential candidates. We demonstrated that epidermal growth factor stimulated ACTH secretion in primates at doses or concentrations equimolar to those of CRH. We also examined the in vivo effects of TNF-α and platelet activating factor (PAF). Both substances produced a CRH-mediated response of the HPA axis of intact rats. We also examined recombinant interleukin-6 (IL-6) in humans. It appears to be the most potent ACTH secretagog known. In a dose- and time-dependent fashion, IL-6 stimulated CRH, and additionally, parvocellular AVP, magnocellular AVP, pituitary ACTH and adrenal cortisol secretion (see project Z01 HD 00615-15 DEB).

In the search of tissue CRF(s) we have examined two models of stress. First, patients undergoing neck exploration or major abdominal surgery and receiving identical perioperative sedation and anesthesia were examined before, during, and after surgery. In the former group we saw only very low plasma concentrations of CRH and mild activation of the HPA axis and the adrenomedullary system during surgery. ACTH and cortisol secretion was continuous rather than pulsatile and the ratio of ACTH to cortisol decreased when compared to basal secretion. These results suggest both the possible presence of a long-acting tissue CRF and of an adrenal component in surgical stress. The major elevation of cortisol, ACTH and epinephrine took place during anesthesia reversal. In contrast, in patients undergoing major surgery there was major activation of the HPA axis both during surgery and during recovery. In these patients, we found elevated concentrations of circulating CRH and profound elevations of AVP, ACTH, cortisol, catecholamines and neuropeptide Y, a peptide which is co-secreted with catecholamines and which has ACTH-like properties. Interestingly, secretion of CRH and ACTH was pulsatile with relative concordance of the pulses which took place approximately every 30-35 min.

Second, we examined the stress of dynamic exercise using a treadmill. Dynamic exercise is a primal component of the "fight or flight" response. The HPA axis as well as vasopressin, growth hormone, aldosterone, and prolactin secretion, are activated during exercise in an exercise intensity-dependent fashion correlating well with both percent of maximal 02 consumption (VO2max) and plasma lactate elevations. Trained subjects could perform high workloads with minimal activation of the axis, a result of chronic adaptation. There was a clearcut relationship between the exercising muscle and the HPA axis.

Upon CRH stimulation highly trained subjects (obligate athletes) had obvious evidence of hypercortisolism (blunted ACTH and cortisol responses and high baseline cortisol) suggesting chronic alterations of their HPA axis reminiscent of those seen in melancholic depression, anorexia nervosa, and chronic active alcoholism. We pursued this lead by psychometric testing of highly trained runners. It appears that during sustained physical training the affect of athletes is normal, suggesting that the self-imposed obligatory exercise in these athletes may be correcting some inherent mood disorder.

To extricate potential differences in the activity of the stress system within the normal population we performed a 90 VO_2max exercise test 2h after administration of a high dose of dexamethasone (4 mg). Two populations of subjects were distinguished. Approximately two thirds of the subjects were completely suppressed, while one-third had marked ACTH, AVP, and cortisol responses, which broke through the suppression of the glucocorticoid. The latter group also scored higher than the former group in the Spielberger anxiety scale. All volunteers had a normal single dose dexamethasone suppression test. The question arises as to whether the latter group is carrying a hereditary and/or constitutional trait of stress system hyperactivity and, hence, increase vulnerability to melancholic depression and anxiety disorders. Could the dexamethasone - exercise test be superior to dexamethasone or oCRH tests in revealing stress system hyperactivity? Could modifications of this combined test also help revealing a hypoactivity group, whose members would be vulnerable to develop atypical depression, chronic fatigue/fibromyalgia syndromes or autoimmune disorders (see project Z01 HD 00615-15 DEB)?

We have developed a hypothalamic organ culture system in which we study the in vitro regulation of hypothalamic CRH secretion directly. We have shown that serotonin, acetylcholine and the catecholamines (alpha receptors) stimulate CRH secretion whereas the GABA/benzodiazepine system is inhibitory. Glucocorticoids, ACTH and β-endorphin, and CRH exert negative feedback (long, and short, and ultra short feedback loops, respectively). Alpha-MSH, and CLIP have suppressing effects on the CRH neuron. Interestingly, epidermal growth factor, interleukin 1, tumor necrosis factor-α, interleukin 2, interferon-γ, platelet activating factor (PAF) and several eicosanoids show strong CRH-releasing properties. The responses to interleukin 1 and TNF-α could be inhibited by prostaglandin synthesis blockade. These substances may represent some of the putative links between the immune-inflammatory response and the endocrine system. Recently, we demonstrated that vasopressin is a potent stimulus of CRH secretion, an effect that could be antagonized with vasopressin receptor antagonists of the V_3 (V_{1B}) type. This is interesting in light of the co-presence of CRH and vasopressin in the paraventricular nucleus and the known synergistic effects of CRH and vasopressin on ACTH secretion.

We have examined the effects of hypothyroidism and hyperthyroidism on the activity of the HPA axis and in particular the CRH neuron. We have found that they are, respectively, associated with hyposecretion and hypersecretion of CRH, a phenomenon that could explain the depressive symptomatology seen in both states. Using similar methodology, we have shown that cholecystokinin stimulates CRH secretion via peripheral vagal receptors and that neuropeptide Y has stimulatory effects upon the HPA axis at two levels: the hypothalamic CRH neuron and the adrenal cortex itself.

Pregnancy is the only known physiologic state in man in which CRH circulates in plasma at levels expected to cause activation of the pituitary adrenal axis (100 pg/ml up to 4000 pg/ml). These levels gradually increase even further during labor. Plasma CRH concentrations return to undetectable (< 15 pg/ml) following delivery. Interestingly, it has been known for years that the last 2-3 months of pregnancy are characterized by hypercortisolism at a degree similar to what has been observed in severe depression and anorexia nervosa. We have developed an in vitro perfusion system in which-full thickness placenta fragments are kept in culture. These fragments contain a 1300 nucleotide long mRNA which hybridizes with a CRH-specific cDNA probe. Glucocorticoids, prostaglandins, catecholamines, oxytocin and vasopressin have no inhibitory or stimulatory effects on placental secretion of CRH in this model. The latter is chromatographically identical to hypothalamic CRH. Our perfused placenta fragments also secrete immunoreactive beta-endorphin, α-MSH, and A-dynorphin, chromatographically identical to synthetic human beta-endorphin, α-MSH, and A-dynorphin, respectively. Both CRH and oxytocin stimulate markedly the secretion of beta-endorphin, α-MSH and A-dynorphin by the placenta. These findings have raised the following questions: 1) Does the high intra-pregnancy CRH suppress hypothalamic secretion of CRH (via cortisol negative feedback) and therefore lead to a brief postpartum adrenal insufficiency state, followed by a later rebound of endogenous hypothalamic CRH secretion 6-8 weeks later? Interestingly, the very common "postpartum blues", occur in the first week postpartum whereas postpartum depression occurs 1-3 months later. 2) What regulates the secretion of placental CRH? Mechanical contraction or ischemia of the placental may be responsible for the elevations of plasma CRH seen during labor. 3) What is the function of pregnancy plasma CRH other than stimulating the maternal pituitary-adrenal axis? Do the vasodilatory properties of CRH play a role in labor? For instance, CRH-induced superior mesenteric vessel dilation may protect the maternal intestinal tract from ischemia. Moreover, circulating CRH might regulate uterine blood vessel flow towards the placenta. The latter was recently shown convincingly by an Australian group.

We recently completed a study examining the circadian and pulsatile activity of the HPA axis in normal pregnant women in the third trimester of pregnancy (32 wks, before the concentrations of CRH-BP in plasma decline). We found the placental CRH is secreted in a pulsatile but noncircadian fashion. Crosscorrelation demonstrated a positive correlation between placental CRH and ACTH and cortisol, however the concurrent presence of the circadian rhythm of ACTH and cortisol suggests that the circadian rhythmicity of ACTH and cortisol are maintained by the other major ACTH secretagog, parvocellular AVP, which is also secreted in a pulsatile and circadian fashion, as parvocellular CRH. Naturally, levels of AVP in the systemic circulation were extremely low, as a result of peripheral proteolysis characteristic of the pregnant state.

We recently completed the analysis of a prospective, longitudinal study of pregnant-postpartum women, which included psychometric testing and CRH testing in the postpartum. Overall, all women were euthymic while pregnant and had a transiently suppressed CRH neuron in the postpartum. When the group that developed blues/depression was compared to the women that remained euthymic, there was a clear difference between the two groups with the former's suppression being more profound and longstanding than the latter's. These findings suggest that the postpartum blues/depression syndromes should be included in the stress system hypofunction states. If women during the postpartum had a defective HPA axis response to inflammatory stimuli, such as IL-6, this would provide an explanation for their increased vulnerability to autoimmune/inflammatory disease during this period of their life (see project Z01 HD 00615-15 DEB).

C. Roles and Actions and Pathophysiology of HPA and HPG Axes Hormones

Glucocorticoids - Glucocorticoid Resistance Syndromes:

Glucocorticoids have multiple actions which are essential in maintenance of homeostasis in both the resting (unstressed) state and during stress. The effects of glucocorticoids that are important on maintenance of resting homeostasis have been called "permissive or normalizing".

Glucocorticoids act upon their target tissues by modulating gene expression. This is attained by the interaction of a glucocorticoid-glucocorticoid receptor complex with specific sites in the nucleus, that regulate the expression of genes coding for function-specific proteins. We had the opportunity to study several large kindreds and individuals with generalized glucocorticoid resistance that had alterations of their glucocorticoid receptors, several New World primate species with generalized glucocorticoid and other steroid hormone resistance, and recently, New World prairie roles with generalized glucocorticoid resistance. We have obtained permanent cell lines from such individuals and species and are studying them in an attempt to understand the molecular mechanisms of hormone resistance and using them as an experiment of Nature to understand fundamental principles of glucocorticoid action. New World primates in addition to glucocorticoid resistance have progesterone, estrogen, aldosterone, androgen and vitamin D resistance. We demonstrated abnormalities in their glucocorticoid, progestin, estrogen and mineralocorticoid receptor. The animal model is of particular interest because its generalized steroid hormone resistance suggests a common link among the six classes of steroid hormones as they interact with their receptor and "acceptor" sites to modulate gene expression.

We have cloned, sequenced and performed in vitro mutagenesis studies with the glucocorticoid receptor of the patients and nonhuman primates. We found two different single amino acid substitutions in the steroid binding domain of the glucocorticoid receptor, which were responsible for the decreased affinity and effect of the glucocorticoid receptor in two different propositi, that of the initial kindred and a subsequent interesting sporadic case. Homozygosity for the defect in the first kindred led to severe disease, whereas heterozygosity was associated with mild hypercortisolism, without overt clinical manifestations. The defect in our sporadic case with glucocorticoid resistance was a novel, de novo nonconservative amino acid substitution in the hinge region the glucocorticoid receptor, which not only abolished all agonist activity of the mutant glucocorticoid receptor, but also caused it to be a potent negative inhibitor of the normal receptor produced by the unaffected allele. The patient had moderate glucocorticoid resistance with hypertension and infertility. In his mid 30's, this patient developed also pituitary Cushing's disease. His corticotropinoma expressed P53 activity, suggesting that decreased glucocorticoid feedback due to glucocorticoid resistance of the corticotroph and of the hypothalamic neurons producing its secretagogs may be an early step in pituitary tumorigenesis. We have several additional families with glucocorticoid resistance whose glucocorticoid receptor we are evaluating now in great detail. Thus, a splice site micro deletion was found, resulting in expression of only one of the alleles, and associated with 50 percent reduction in receptor number and hyperandrogenism in the proposita of this kindred and biochemical hypercortisolism in the affected brothers and father. Our results indicate that glucocorticoid resistance results from multiple defects and can be autosomal recessive, with heterozygotes expressing a subclinical phenotype, or autosomal dominant, with heterozygotes presenting with disease. We also have 2 families in whom we found no structural abnormality of the glucocorticoid receptor gene. We consider alternative mechanisms, such as changes in the expression or function of heat shock protein 90.

We obtained additional evidence that glucocorticoid resistance could lead to corticotropinoma formation. One of 4 patients with Nelson's syndrome was found to have a somatic nonsense mutation resulting in a frame-shift and a truncated, inactive glucocorticoid receptor. Interestingly, none of the 4 tumors expressed abnormal P53 activity.

Glucocorticoids- An Endogenous Determinant of Glucocorticoid Sensitivity:

The human glucocorticoid receptor gene, located in the long arm of chromosome 5 consists of 10 exons. Exons 9α and 9β can be alternatively spliced into the mRNA to encode for the classic glucocorticoid receptor α and a nonligand-binding receptor which differs only in the C terminus. We demonstrated that mRNA of the β isoform is expressed in all human tissues examined and that in a co-transfection system in vitro it is able to antagonize the effects of glucocorticoids mediated by the α isoform. We concluded that the ratio of GRα to GRβ may determine the sensitivity of a tissue to glucocorticoids.

Glucocorticoids - Effects on Growth:

We examined the influence of hypercortisolism on basal and stimulated growth hormone (GH) secretion. Young patients with Cushing's syndrome have profoundly suppressed spontaneous and provoked GH secretion. This deficiency takes over a year to correct, suggesting a rather protracted direct or indirect effect of the hypercortisolism on the somatotroph. Interestingly, during the convalescence year, levels of insulin-like growth factor I (IGF-I) in plasma remain normal, while those of IGF-binding protein 3 decrease, providing an explanation for the continuing growth of pediatric patients after cure of hypercortisolism, even though the levels of GH are suppressed. We found that patients who had Cushing's syndrome during their growth years lost on the average 7 cm of their final height, when they were followed up as adults.

Glucocorticoids - HPA Axis Feedback Effects via Type I Receptors:

The glucocorticoid receptor type I (mineralocorticoid receptor) is profoundly involved in the feedback regulation of the HPA axis in the rat. We demonstrated that the mRNA of this peptide is also expressed in Old and New World primates, in similar regions of the CNS. We examined its importance by administering specific type I antagonists to normal humans but found no effect of such antagonists on basal and CRH-stimulated cortisol secretion.

Aldosterone Resistance-Pseudohypoaldosteronism:

Pseudohypoaldosteronism is a congenital condition characterized by profound salt loss, hypokalemia and acidosis in the presence of extremely elevated levels of plasma and urinary aldosterone. The resistance to aldosterone can be limited to the kidney or generalized to all mineralocorticoid target tissues. The clinical severity of the syndrome improves with age. About 2/3 of the cases are sporadic, while 1/2 are familial. We studied several sporadic cases for the presence of defects in the mineralocorticoid receptor to find no pathogenetic mutations. We have focused our efforts on a postreceptor mechanism, and have already found mutations in one of the subunits of the amiloride-sensitive sodium channel, the γ-subunit.

ACTH Resistance - Hereditary Isolated Glucocorticoid Deficiency:

ACTH regulates glucocorticoid secretion by the adrenal cortex via its recently cloned receptor. This membrane protein belongs to the superfamily of G protein-coupled receptors, forming its own distinct class with the receptors for αMSH. We mapped the gene of the ACTH receptor in the short arm of chromosome 18 and studied families with hereditary isolated glucocorticoid deficiency (congenital insensitivity to ACTH) for potential defects of this gene. We found that point mutations resulting in premature termination of the receptor or nonconservative amino acid substitutions in key functional areas of the molecule cause the disease. Heterozygote carriers are clinically healthy but revealed by CRH testing, since they have markedly exaggerated responses of ACTH to this hormone.

Testicular LH Resistance - Leydig Cell Hypoplasia - Ovarian LH Resistance:

LH regulates testosterone secretion by Leydig cells and ovulation and corpus luteum formation by the ovarian follicle via its recently cloned receptor. This membrane protein belongs to the superfamily of G protein coupled receptors, forming its own distinct class with the receptors for the other glycoprotein hormones. This group studied families with hereditary primary Leydig cell failure with genetic male members that presented with phenotypically female external genitalia or micropenis and genetic females with primary amenorrhea and infertility and found that point mutations resulting in premature termination of the receptor or nonconservative amino acid substitutions in key functional areas of the receptor molecule caused the disease. Heterozygotes were clinically healthy. Genetic females with complete absence of the LH receptor developed normally in utero and had a normal pubertal development, but remained anovulatory.

Excessive Aromatase Syndrome:

Estrogen is formed by aromatization of androgen within or outside the gonads, primarily in peripheral adipose tissue. We have described a large, 3 generation kindred with excessive extragonadal aromatization, presenting as prepubertal gymecomastia in males and precocious puberty and massive macromastia in females. We have isolated a novel aberrant promoter of the aromatase gene leading to excessive transcription and, hence, production of the enzyme.

D. Mechanisms of Adrenocortical Tumorigenesis

We have studied plausible mechanisms of adrenocortical tumorigenesis with interesting preliminary results. All tumors are monoclonal. About one third of malignant adrenocortical tumors and both available adrenocortical cancer cell lines have abnormalities of the p53 gene, while none, including the two cell lines and a series of adenomas has activating mutations of the ACTH receptor gene or mutations of the G proteins.

In our patient population, we had 5 kindreds and several sporadic cases with primary pigmented nodular adrenocortical disease (PPNAD) or micronodular adrenal disease, a major component of Carney complex, a multiple neoplasia/lentiginosis syndrome transmitted in an autosomal dominant fashion. In collaboration with J.A. Carney of the Mayo Clinic and further recruitment, we included an additional 9 kindreds in our series and attempted chromosomal mapping of the syndrome by linkage analysis. We indeed mapped the defective gene of the complex within a ~4 cM region of the short arm of chromosome 2 in band p16. A small number of genes are known in this area, such as those of calcineurin B and two DNA repair enzymes, previously shown to be involved in nonpolyposis colon cancer and/or microsatellite instability.

Significance to Biomedical Research and the Program of the Institute:

A. Clinical Applications and Implications of CRH

Different patterns of plasma ACTH and cortisol responses to CRH have been noted in different diseases, including hypercortisolism and hypocortisolism. The CRH stimulation test appears to differentiate between Cushing's disease and the ectopic ACTH syndrome. In addition, it appears to be of diagnostic value in distinguishing between Cushing's disease and hypercortisolism due to depression. Because of its biological properties hCRH is an important means to study the normal physiology of the hypothalamic-pituitary-adrenal axis. In psychiatric diseases characterized by hypercortisolism, such as melancholic depression and anorexia nervosa, a pathophysiologic role has been suggested for CRH and this hypothesis is currently tested. Were it true, new treatments could be developed with major goal to prevent CRH hypersecretion or action. We plan to examine the efficacy of a newly discovered nonpeptide CRH antagonist, which could be used orally and which is expected to cross the blood brain barrier.

Our recent studies with atypical depression, the chronic fatigue/fibromyalgia syndromes and the postpartum blues/depression syndromes suggest that a spectrum of human pathologic conditions exist in which the central CRH system is hypofunctional. Activation of the CRH system in such patients may help with the resolution of the clinical picture. Also, CRH agonists or molecules that displace CRH from its binding protein might be useful in the treatment of these conditions. We study the psychological, social and cognitive changes in patients with Cushing's syndrome before and after cure of their hypercortisolism. It is of interest that we have found similarities between Cushing's syndrome both before and after cure and atypical depression, probably stemming from the same pathologic decrease in CRH secretion. We found that the common marmoset could be an excellent model of human depression and psychosocial retardation. We found that sexual abuse in childhood has profound prolonged effects on the hypothalamic-pituitary-adrenal axis, as well as on the affect of these individuals.

B. Regulation of the Hypothalamic-Pituitary-Adrenal Axis In Vivo and In Vitro

We determined the changes of the HPA axis during the stress of surgery or exercise. We have also demonstrated that hyperthyroidism and hypothyroidism are respectively, associated with hypersecretion and hyposecretion of CRH which might explain the presence of depressive symptomatology in both conditions. Several peripheral substances secreted during stress (interleukin 1 and 6, TNF-α, epidermal growth factor, platelet activating factor, prostanoids) appear to be CRH secretagogs of physiologic relevance. We determined the major stimulatory (serotonin, acetylcholine, norepinephrine) and inhibitory (GABA/benzodiazepine system) neurotransmitters of hypothalamic CRH secretion in vitro and demonstrated the presence of a long, a short and an ultra-short feedback loop respectively with glucocorticoids, ACTH and β-endorphin, and CRH, all playing a role at the level of the CRH neuron. These advances from basic science are helpful in understanding the various disorders of the HPA axis that we study. We have demonstrated that the human placenta secretes large quantities of CRH, POMC-derived peptides (primarily β endorphin and α-MSH) and dynorphin A and that CRH exerts autocrine and/or paracrine actions on the placenta by causing β-endorphin, α-MSH and dynorphin A secretion by this tissue. These results may influence our way of thinking about the physiology and pathophysiology of pregnancy and labor and delivery. We confirmed our hypothesis that the postpartum blues/depression represent a state of "adrenal suppression" characterized by suppression of hypothalamic CRH secretion and symptoms of atypical depression. We have examined in great detail the regulatory region of the human CRH gene.

C. Roles, Actions, and Pathophysiology of HPA and HPG Axis Hormones

Pathologic mutations and/or deletions of the glucocorticoid receptor gene are responsible for human familial glucocorticoid resistance. Generalized or tissue-specific resistance can lead to human tumorigenesis. The ratio of the two isoforms of the glucocorticoid receptor may define a tissue's sensitivity or resistance to glucocorticoidds. The glucocorticoid receptor isoforms may participate in the translocation of AIDS virus proteins, such as vpr, which by activating the glucocorticoid receptor may stimulate viral replication via the GREs present in the promoter region of HIV. Also, by exerting glucocorticoid effects, the glucocorticoid receptor - vpr complex may cause further immunosuppression. Thus, the glucocorticoid receptor may participate in the hallmark manifestations of AIDS, may hasten the conversion from the carrier to the disease state and may curtail the terminal phase of the disease.

A transcription or other factor common to all steroid hormones may be defective in New World primates. We suggest that the glucocorticoid receptor may be involved in highly prevalent human diseases, such as obesity, hypertension, depressive/anxiety syndromes and autoimmune diseases by providing excessive or defective glucocorticoid activity to a tissue.

It is important to pursue our search for the molecular defect of pseudohypoaldosteronism. We have defined common human mineralocorticoid receptor and amiloride-sensitive sodium channel subunit α polymorphisms which may be useful in the pursuit of the genetic determinants of hypertension.

We have examined several families with hereditary isolated glucocorticoid deficiency (ACTH resistance) for possible defects of the ACTH receptor gene. We found pathologic mutations, altering the receptor and causing the disease. The heterozygote parents and grandparents have abnormal ACTH responses to ovine corticotropin releasing hormone, suggesting that this test can be employed for the ascertainment of the carrier state in this disorder. We found that the triple A syndrome, which has hereditary isolated glucocorticoid deficiency as a component (Adrenal insufficiency, Achalasia, Alacrima), is not associated with molecular defects of the ACTH receptor.

We have defined the LH receptor gene as a locus of the defect in complete male pseudohermaphroditism (Leydig cell hypoplasia) and micropenis in genetic males and in anovulation and primary amenorrhea in genetic females.

We have defined the aromatase gene as the locus of the defect in the syndrome of excessive aromatization, and determine that the syndrome can present as precocious puberty and massive macromastia in females.

D. Mechanisms of Adrenocortical Tumorigenesis

Adrenal cancer continues to represent a rare but aggressive carcinoma that affects children and young individuals and responds little in terms of medical therapy. Every effort should be placed in understanding this disease, in achieving early diagnosis and in devising effective cures.

Our Carney complex research may not only elucidate the molecular pathophysiology of a rare multiple neoplasia/lentiginosis syndrome that afflicts children and young individuals but also may help unravel important physiological processes and mechanisms of tumorigenesis, as it has happened already with other rare syndromes.

Proposed Course:

A. Clinical Applications and Implications of CRH

1. *Is CRH Involved in the Pathophysiology of Affective Disorder?*

a) We have formulated the general hypothesis that CRH plays a pathophysiologic role in psychiatric disease characterized by hypersecretion or hyposecretion of this peptide. CRH antagonists or inhibitors of CRH secretion as well as CRH agonists or stimulants of CRH secretion, will be used as tools to test this hypothesis. Animal models for depression would be ideal to begin such testing.

b) It is evident from studies by S. Levine and S. Suomi that neonatal separation of rats or monkeys from their mothers can be associated with hyperactivity of the hypothalamic-pituitary-adrenal axis and behavioral disturbances throughout their life. We obtained similar results in sexually abused girls long after the abuse took place. These findings suggest that there are critical periods in human life as well, associated with permanent alterations in the reactivity to stress. The hypothesis that CRH is involved in this early sensitization can be examined in various ways. We plan to use CRH antagonists to reverse the clinical picture of animals that develop the syndrome.

2. *Can We Unravel Affective Disorder Traits?*

If for genetic or constitutional reasons the number of hypothalamic CRH neurons in patients prone to develop melancholic depression/anxiety disorders were increased, one would hope that a strong stimulus of the CRH neuron, such as IL-6, would be able to distinguish this group from normal controls. The same may be true for our dexamethasone-exercise test paradigm. Similarly, if for the same reasons the number of hypothalamic CRH neurons in patients prone to develop atypical depression, the chronic fatigue/fibromyalgia syndrome or autoimmune disease, were decreased, IL-6 might also be able to detect them.

3. *Differential Diagnosis of Cushing's Disease from Pseudo-Cushing States*

In spite of the assistance of the combined dexamethasone suppression/oCRH stimulation test, it is still difficult to differentiate some patients with depression and hypercortisolism from patients with mild Cushing's disease. Based on previously obtained information from human and in vivo and in vitro studies, we have designed a study which may assist us further with this differential.

Thus, the cytokine IL-6 administered acutely should stimulate the HPA axis of patients with pseudoCushing's (depression, chronic active alcoholism) but not that of patients with Cushing's syndrome of any etiology since in all instances hypothalamic CRH should be suppressed.

4. *Postpartum "Blues" and Postpartum Depression*

Following delivery of the fetus and the placenta, the mother loses a source of peripheral CRH production. This is likely to be associated with subphysiological cortisol secretion during the first days of the postpartum period, a result of a suppressed hypothalamic CRH neuron. Interestingly, 40-50% of postpartum women develop the postpartum blues during the first 2-3 weeks postpartum. We hypothesized that this syndrome was equivalent to glucocorticoid withdrawal, in a sense being an analogue of the steroid withdrawal syndrome of the post transsphenoidal surgery patient with Cushing's disease, who despite glucocorticoid replacement develops "postoperative blues".

Three weeks to three months postpartum, approximately 10% of women develop a frank form of depression which interferes with their overall function. Interestingly, this is the period of the putative hypothalamic CRH recovery. Our prospective study showed the alterations of the hypothalamic-pituitary-adrenal axis in the postpartum and the suppression observed was related to mood changes in the mothers. We anticipate that high levels of plasma CRH or high urinary free cortisol excretion pre-partum might predict the severity of the postpartum blues or depression syndrome. A larger study needs to be performed now to test this hypothesis. In a small subgroup, IL-6 should be administered, as discussed in project Z01 HD 00615-15 DEB, to test the hypothesis of diminished responsiveness to inflammatory stimuli in this period, which is characterized by increased vulnerability to autoimmune disease.

Growth and development of infants of postpartum depressed mothers might be affected to a degree proportional to the severity of the maternal syndrome. Interestingly, maternal circulating CRH and cord blood CRH concentrations are positively correlated. Thus, the infants should be followed as well.

B. Regulation of the Hypothalamic-Pituitary-Adrenal Axis In Vivo and In Vitro

1. *Regulation of the Circadian Rhythmicity and Stress Activation of the Human and Rat Hypothalamic-Pituitary-Adrenal Axis*

We have shown that continuous i.v. infusion of pharmacologic amounts of oCRH in normal volunteers leads to mild hypercortisolism (UFC 150-200 mcg/d) and preservation of the plasma ACTH and cortisol circadian rhythm. Interestingly, the secretory episodes of ACTH and cortisol continue to be seen despite the maximal circulating concentration, of CRH. On the other hand, CRH concentrations in the CSF have a circadian rhythm which is in opposite phase to the circadian rhythm of plasma cortisol concentrations. We have postulated that ACTH secretagogues other than CRH may be involved in the regulation of episodic secretion of ACTH and cortisol and the circadian rhythmicity of the HPA axis. Vasopressin is a such potential factor. We have available several (V_3) receptor vasopressin antagonists which will be administered continuously i.v. to test the hypothesis that vasopressin participates in the generation of the circadian rhythm. We have also generated antibodies against vasopressin and CRH with which we perform immunoneutralization studies in experimental animals.

The V_3 receptor antagonists could be employed to define the degree of vasopressin participation in the stress-related activation of the pituitary-adrenal axis in man. Thus, graded levels of exercise, or an insulin tolerance test, could take place while a vasopressin antagonist infusion is being administered.

2. *Regulation of Placental CRH, POMC-Derived Peptides and Dynorphin A In Vivo and In Vitro*

The possible association of placental CRH secretory episodes with uterine contractions will be examined by frequent, every 3 min serial sampling over 3 hours in end-stage labor.

Prostanoids and platelet activating factor will be examined as stimulants of placental CRH or POMC-derived peptides in vitro. Of particular interest are also several agents, including serotonin, epidermal growth factor (EGF), platelet derived growth factor (PDGF), interleukins 1 and 6, and tumor necrosis factor (cachexin), which cause hypothalamic CRH secretion in vitro. PDGF was recently found to mimic the effect of serum from preeclamptic women in causing lysis of cultured endothelial cells. It is of interest that preeclampsia is associated with elevated circulating CRH concentrations in pregnancy.

C. Roles, Actions and Pathophysiology of HPA and HPG Axes Hormones

1. *Cloning of the Glucocorticoid Receptor Type II Gene in Patients with Glucocorticoid Resistance and in Animal Models*

We are now examining the glucocorticoid receptor of members of several new families with glucocorticoid resistance. Also, we are pursuing several hypotheses for the mechanism(s) of glucocorticoid resistance in the 2 families in which no structural abnormalities of the glucocorticoid receptor gene were found. The aldosterone, progesterone, and estrogen receptor of the New World primates will be cloned also, with the assistance of probes obtained from the human. We postulate that the common link between all steroid hormones that is responsible for the generalized steroid hormone resistance in New World primates represents a common modulator of all steroid receptors. This could be an enzyme, such as a steroid receptor phosphokinase, a common trans-activating factor necessary for the interaction of the steroid receptor in the nucleus with their responsive elements, or a common steroid-class specific binding protein. We are characterizing the prairie vole as a glucocorticoid resistant animal and examine the molecular mechanisms of resistance in this species, as well.

2. *Continuing Studies with the β-Isoform of the Glucocorticoid Receptor*

There are many important questions remaining in this area. We need to demonstrate and quantify the actual protein content of each isoform in human tissues. We need to demonstrate the actual location of the β isoform within the cell and its relation to heat shock proteins. Preliminary studies have suggested that it is cytosolic and bound to heat shock protein 90 and that, upon activation of the α-isoform by glucocorticoid, it forms heterodimers, which putatively have decreased transactivational and transrepressive activities. We need to examine the interaction of the β isoform not only with GREs, but also with transcription factors known to interact with glucocorticoid receptors and known to be influenced by this interaction. These include the cjun-cfos and the NF-kB/Rel A heterodimers. One could include here the HIV protein vpr, which apparently in some tissues potentiates the glucocorticoid receptor in both enhancing viral replication and suppressing host immune functions, while in other tissues it causes glucocorticoid resistance. We will pursue also the potential involvement of GRβ in human pathophysiology. Thus, we will examine tissues of patients with glucocorticoid-resistant asthma and other conditions for overexpression of the β-isoform, and tissues of patients with glucocorticoid hypersensitivity (metabolic syndrome X) for underexpression of the β-isoform.

3. *Molecular Mechanisms of Aldosterone Resistance*

We are pursuing a likely postreceptor site, the amiloride-sensitive sodium channel, with its 4 known subunits.

4. *ACTH Receptor Studies in Patients with ACTH Resistance*

We are now examining the ACTH receptor in several additional kindreds with isolated hereditary ACTH resistance, as well as kindreds with the triple A syndrome (Adrenal insufficiency, Achalasia, Alacrima). In the latter we will first attempt genetic linkage studies, once we have collected the necessary number of subjects.

5. *LH Receptor Studies in Patients with LH Resistance*

We are examining other potential candidates with defects of the LH receptor.

D. Mechanisms of Adrenocortical Tumorigenesis

We continue to examine major candidate genes potentially participating in adrenocortical tumorigenesis. These include those of p16, steroidogenic factor 1 and DAX 1.

We continue our two-prong approach towards elucidating the molecular pathophysiology of Carney complex. Thus, we examine candidate genes within the 2p16 region, in which we localized the gene and continue to cone down on a progressively smaller region, until we hopefully identify the actual gene.

Protocols:

Human

82-CH-45	Chrousos	Dose-Response Relationship for Single Doses of Corticotropin-Releasing Hormone (CRH) in Normal Volunteers and in Patients with Adrenal Insufficiency (active) This blanket protocol enables us to administer ovine corticotropin-releasing hormone (CRH) as a provocative test of the hypothalamic-pituitary-adrenal (HPA) axis in men, women, and children with potential alterations of this axis. The peptide continues to be an investigational drug (IND# 19802) and all data concerning its administration are captured and reported annually to the FDA. During the past year oCRH was administered under this and other approved protocols.
82-CH-125	Gold	Studies of Corticotropin Releasing Factors (CRF) and Related Neurotransmitters, Neurotransmitter Metabolites and Peptides in the Spinal Fluid of Patients with Cushing's Disease (completed)
86-CH-172	Laue	Dose Response Relationships between Exogenous ACTH and Adrenal Cortisol Secretion in Normal Volunteers and Patients with Abnormalities of the Hypothalamic-Pituitary-Adrenal Axis (completed)
88-CH-120	Chrousos	The Hypothalamic-Pituitary-Adrenal Axis in Pregnancy, the Postpartum Period, and in Postpartum Depression Syndromes (completed)

88-CH-115	Chrousos	Placental Secretion of Corticotropin Releasing Hormone during Pregnancy and Labor and Delivery (partially completed)
90-CH-125	Chrousos	Psychological, Social and Cognitive Changes in Children, Adolescents and Adults with Cushing syndrome (completed)
90-CH-124	Friedman	Circulating Hormones and EEG Monitoring in Patients with Cushing's Syndrome (completed)
90-CH-194	Yanovski	Inferior Petrosal Sinus Sampling for the Determination of ACTH Concentration in Normal Volunteers and in Patients with Disorders of the Hypothalamic-Pituitary-Adrenal Axis (active) This protocol allows a procedure which is diagnostic in Cushing's syndrome to be performed in normal volunteers and patients with abnormalities of the HPA axis, whom it would not benefit. This protocol, thus, provides valuable normal control samples and samples that may help us understand better the pathophysiology of the HPA axis.
91-CH-166	Chrousos	Hypothalamic-Pituitary-Adrenal Axis Function in Offspring of Affectively Ill Mothers vs Offspring of Normal Control Mothers (completed)
92-CH-181	Peeke	Energy Metabolism in Patients with Seasonal Affective Disorder (completed)
94-CH-134	Papanicolaou	Dose-Response Relationships for Single Doses of Recombinant Interleukin-6 in Normal Volunteers and Patients with Disorders of the Hypothalamic-Pituitary-Adrenal Axis (active) This protocol concerns the administration of recombinant interleukin-6 to normal volunteers and patients with disorders of the HPA axis. Interleukin-6 is an investigational drug (IND# 5419), with which we have already obtained some experience from a phase 1 study of cancer patients done by NCI. A dose-response curve in normal volunteers has been completed and a dose has been selected to be administered to the various patient groups listed in the protocol. The hypothesis of the protocol is that as a hypothalamic stimulus of CRH, acute administration of interleukin-6 will distinguish biochemically not only patients with a clearly disordered HPA axis, i.e. Cushing's vs. pseudoCushings's vs. melancholic depression vs. chronic active alcoholism or melancholic vs. atypical depression, but also premorbid states of vulnerability, i.e. individuals prone to melancholic depression or anxiety disorders or individuals prone to atypical depression, chronic fatigue/fibromyalgia, postpartum blues or depression, alcoholism or autoimmune disorders.
95-CH-59	Stratakis	Definition of the Genotype and Clinical Phenotype of Primary Pigmented Adrenocortical Disease and Its Associated Conditions (Carney Complex) (active)

This protocol allows the examination and obtaining of samples from patients with Carney Complex and their families. Samples include blood for leukocytes and DNA and tumors for cytogenetic and DNA studies. The first phase of the protocol, to collect sufficient numbers of samples to perform the initial mapping of the defective gene by linkage analysis has been completed. More precise localization and testing of candidate genes and examination of tumors for clues continue.

Animal

88-011	rat	Chrousos	Effect of Thyroid Hormones on Hypothalamic-Pituitary-Adrenal Function (completed)
89-036	rat	Chrousos	Regulation of Hypothalamic CRH & Pituitary ACTH Release (active)
91-025	rat	Chrousos	Heat Shock Protein 90 (HSP 90) and Glucocorticoid Receptor Gene Expression In Vivo: Effects of Adrenalectomy and Glucocorticoid Administration (completed)
92-031	rat	Chrousos	The Effects of Acute and Chronic Stress on Hypothalamic-Pituitary-Adrenal Axis and Sympathetic Nervous System Function of the Zucker (fa/fa) Rat (completed)
93-037	rat	Cizza	Regulation of the Hypothalamic-Pituitary-Adrenal Axis at Basal Conditions and During Immobilization in the Aged Male and Female Fischer 344/N Rat (active)
69-005	rat	Chrousos	Characterization of Nonpeptide CRH Receptor Antagonist Analogs

Publications:

Arai K, Tsigos C, Suzuki Y, Listwak S, Zachman K, Zangeneh F, Rapaport R, Chanoine JP, Chrousos GP. No apparent mineralocorticoid receptor defect in a series of sporadic cases of pseudohypoaldosteronism. J Clin Endocrinol Metab 1995;80:814-7.

Bamberger CM, Bamberger AM, Wald M, Chrousos GP, Schulte HM. Inhibition of mineralocorticoid activity by the β-isoform of the human glucocorticoid receptor. J Steroid Biochem Mol Biol, in press.

Bamberger CM, Bamberger AM, Chrousos GP. Dominant negative inhibitors of the glucocorticoid receptor. In: De Bellis A, Schipani E, eds. Frontiers in Endocrinology, Vol. 14. Serono Symposia Publication, Rome, Italy, 1995;129-39.

Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity. Endo Rev 1996;17:221-44.

Burgess ES, Dorn LD, Haaga D, Chrousos GP. Sociotropy, autonomy, stress and depression in cushing syndrome. J Nerv Ment Dis, in press.

Carter CS, DeVries AC, Taymans SE, Roberts RL, Williams JR, Chrousos GP. Adrenal corticoid hormones and the development and expression of mammalian monogamy. Ann NY Acad Sci 1995;771:82-91.

Chrousos GP, Castro M, Leung DYM, Webster E, Kino T, Bamberger CM, Elliot S, Stratakis C, Karl M. Molecular mechanisms of glucocorticoid resistance/hypersensitivity. Am J Resp Crit Care Med, in press.

Chrousos GP, McCarty R, Cizza G, Pacak K, Sternberg E, Gold PW, Kvetnanski R. Stress: mechanisms and clinical implications. Ann NY Acad Sci 1995;771, New York. (755 pages, ISBN 0-89766-943-6, cloth; 0-89766-944-4, paper)

Cizza G, Nieman LK, Doppman JL, Passaro MD, Czerwiec FS, Chrousos GP, Cutler GB Jr. Factitious cushing syndrome. J Clin Endocrinol Metab, in press.

Cizza G, Gold PW, Chrousos GP. Aging is associated in the 344/N fischer rat with decreased stress responsivity of central and peripheral catecholaminergic systems and impairment of the hypothalamic-pituitary-adrenal axis. Ann NY Acad Sci 1995;771:491-511.

Dorn LD, Burgess ES, Susman EJ, Von Eye A, DeBellis MD, Gold PW, Chrousos GP. Response to ovine corticotropin releasing hormone in depressed and nondepressed adolescents: does gender make a difference? J Am Acad Child Adolesc Psychiatry, in press.

Friedman TC, Nieman LK, Yanovski JA, Doppman JL, Cutler GB Jr., Oldfield EH, Chrousos GP, Kalogeras KT. Inferior petrosal sinus arginine vasopressin concentrations in normal volunteers and patients with cushing disease. J Clin Endocrinol Metab, in press.

Friedman TC, Mastorakos G, Newman TD, Mullen NM, Horton EG, Costello R, Papadopoulos NM, Chrousos GP. Carbohydrate and lipid metabolism in endogenous hypercortisolism: shared features with metabolic syndrome X and noninsulin-dependent diabetes mellitus. Endocrine Journal (Japan).

Gold PW, Licinio J, Wong ML, Chrousos GP. Corticotropin releasing hormone in the pathophysiology of melancholic and atypical depression and in the mechanism of action of antidepressant drugs. Ann NY Acad Sci 1995;771:716-29.

Johnson EO, Decker L, Gold PW, Chrousos GP. Distribution of hippocampal mineralocorticoid and glucocorticoid receptor mRNA in a glucocorticoid resistant nonhuman primate. Steroids 1996;61:69-73.

Johnson EO, Kamilaris TC, Calogero AE, Gold PW, Chrousos GP. Effects of early parenting on growth and development in a small primate. Ped Res 1996;39:999-1005.

Johnson EO, Kamilaris TC, Carter CS, Calogero AE, Gold PW, Chrousos GP. The biobehavioral consequences of psychogenic stress in a small, social primate (*callithrix jacchus jacchus*). Biol Psychiatry

Kalogeras KT, Nieman LK, Friedman TC, Dopppman JL, Cutler GB Jr., Chrousos GP, Wilder RL, Gold PW, Yanovski JA. Inferior petrosal sinus sampling in normal human subjects reveals a unilateral corticotropin-releasing hormone-induced arginine vasopressin release associated with ipsilateral adrenal corticotropin secretion. J Clin Invest 1996;97:2045-50.

Karl M, Von Wichert G, Kempter E, Katz DA, Reincke M, Monig H, Ali IU, Stratakis CA, Oldfield EH, Chrousos GP, Schulte HM. Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab 1996;81:124-9.

Karl M, Saviolakis GA, Gravanis A, Chrousos GP, Margioris AN. The PC 12 rat pheochromocytoma cell line expresses the prodynorphin gene and secretes the 8 kdalton dynorphin product. Regulatory Peptides 1996;61:99-104.

Karl M, Lamberts SWJ, Koper JW, Katz DA, Huizenga NE, Kino T, Haddad BR, Hughes MR, Chrousos GP. Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assn Amer Phys, in press.

Karl M, Chrousos GP. Experimental and clinical models of CRH-induced pituitary tumors. In: Oncogenesis and molecular biology of pituitary tumors. Melmed S, ed. Front Horm Res Karger, Basel, 1996;20:54-71.

Latronico AC, Anasti J, Arnhold IJP, Rapaport R, Mendonca BB, Bloise W, de Castro M, Tsigos G, Chrousos GP. Testicular and ovarian resistance to luteinizing hormone caused by homozygous inactivating mutations of the luteinizing hormone receptor gene. N Engl J Med 1996;334:507-12.

Leong GM, Mercado-Asis LB, Reynolds JC, Oldfield EH, Chrousos GP. The effect of cushing disease on bone mineral density, body composition, growth and puberty: a report of an identical adolescent twin pair. J Clin Endocrinol Metab 1996;81:1905-11.

Magiakou MA, Mastorakos G, Rabin D, Dubbert B, Gold PW, Chrousos GP. Hypothalamic corticotropin releasing hormone suppression during the postpartum period: implications for the increase of psychiatric manifestations during this time. J Clin Endocrinol Metab 1996;81:1912-7.

Magiakou MA, Mastorakos G, Rabin D, Margioris AN, Dubbert B, Calogero AE, Tsigos C, Munson PJ, Chrousos GP. The Maternal hypothalamic-pituitary-adrenal axis in third trimester human pregnancy. Clin Endocrinol 1996;44:419-28.

Magiakou MA, Chrousos GP. Glucocorticoid therapy and withdrawal. Chapter 6 In: RC Bone. Current practice of medicine. Current medicine, inc., Philadelphia, 1996;VI 6.1-6.

Marini JC, Bordenick S, Chrousos GP. Endocrine aspects of growth deficiency in OI. Connective tissue research 1995;31:S55-7.

Mastorakos G, Patchev VK, Chader GJ, Chrousos GP, Gaudet SJ. Differences in arylakylamine N-acetyltransferase activity between inflammatory disease-susceptible lewis and -resistant fischer rats. Neuroendocrinology 1995;62:71-8.

Mastorakos G, Scopa CD, Kao LC, Vryonidou A, Friedman TC, Kattis D, Phenekos C, Rabin D, Chrousos GP. Presence of immunoreactive corticotropin releasing-hormone (CRH) in human endometrium. J Clin Endocrinol Metab 1996;81:1046-50.

Mastorakos G, Chrousos GP. Chapter 79, Adrenal hyperandrogenism. In: Reproductive endocrinology, surgery, and technology. Adashi E, Rock J, Rosenwaks Z, eds. Raven Press, New York, 1995;1539-1553.

Pacak K, McCarty R, Palkovitz M, Cizza G, Kopin IJ, Goldstein D, Chrousos GP. Decreased central and peripheral catecholaminergic activation in obese zucker rats. Endocrinology 1995;136:4360-7.

Peeke PM, Chrousos GP. Hypercortisolism and obesity. Ann NY Acad Sci 1995;771:665-76.

Premkumar A, Stratakis CA, Shawker TH, Papanicolaou DA, Chrousos GP. Testicular ultrasound in carney complex: report of three cases. J Clin Ultrasound

Stratakis CA, Mitsiadis NS, Sun D, Chrousos GP, O'Connell A. Recurring oral giant cell lesion in X-linked hypophosphatemic rickets: clinical manifestation of occult hyperparathyroidism? J Peds 1995;127:444-6.

Stratakis CA, Carney A, Ping J, Papanicolaou DA, Karl M, Kastner DL, Pras E, Chrousos GP. Carney complex, a familial multiple neoplasia and lentiginosis syndrome: analysis of 11 kindreds and linkage to the short arm of chromosome 2. J Clin Invest 1996;97:699-705.

Stratakis CA, Mitsiades NS, Chrousos GP, Margioris AN. Dopamine affects rat placenta opioids in a receptor- and opioid type-specific manner. Eur J Pharmacol, in press.

Stratakis CA, Jenkins RB, Pras E, Mitsiadis CS, Raff SB, Stalboerger PG, Tsigos C, Carney A, Chrousos GP. Cytogenetic and mircosatellite alterations in tumors from patients with the syndrome of myxomas, spotty skin pigmentation, and endocrine overactivity (carney complex). J Clin Endocrinol Metab, in press.

Stratakis CA, Vamvakopoulos NC, Chrousos GP. Corticotropin-releasing hormone, stress and depression. In: Bailly D, ed. Neuroendocrine aspects of mood disorders. Ardix médical, Levallois 1995;119-52 (in french).

Stratakis CA, Chrousos GP. Neuroendocrinology and pathophysiology of the stress system. Ann NY Acad Sci 1995;771:1-18.

Taymans SE, DeVries AC, DeVries MB, Nelson RJ, Friedman TC, de Castro M, Detera-Wadleigh S, Carter CS, Chrousos GP. The prairie vole (*microtus ochrogaster*) as a model of glucocorticoid resistance. Gen Comp Endocrinol

Tsigos C, Chrousos GP. Stress, endocrine manifestations and diseases. Chapter 3. In: Handbook of stress medicine. Cooper CL, ed. Boca Raton, FL: CRC Press Inc., 1995;61-65.

Tsigos C, Arai K, Latronico AC, Webster E, Chrousos GP. Receptors for melanocortin peptides in the hypothalamic-pituitary-adrenal axis and skin. Ann NY Acad Sci 1995;771:352-63.

Tsigos C, Chrousos GP. The neuroendocrinology of the stress response. Chapter 6. In: NIDA alcohol research monograph: stress, gender, and alcohol seeking behavior. Hunt WA, Zachari S, eds. CSR, Inc., Washington, DC, 1995;29:103-24.

Tsigos C, Kamilaris T, Chrousos GP. Adrenal diseases. In: Moore WT, Eastman R, eds. Diagnostic endocrinology, 2nd edition. B.C. Decker Inc., Toronto, 1996;123-156.

Tsigos C, Chrousos GP. Differential diagnosis and management of cushing's syndrome. Ann Rev Med 1996;47:443-61.

Wong M, Ramayya MS, Chrousos GP, Driggers PH, Parker KL. Cloning and sequencing analysis of the human gene encoding steroidogenic factor 1. J Mol Endocrinol, in press.

Yanovski JA, Yanovski SZ, Harrington L, Gold PW, Chrousos GP. Differences in the hypothalamic-pituitary-adrenal axis of black and white men. Hor Res 1995;44:208-12.

Yanovski JA, Zelitch-Yanovski SA, Friedman TC, Loh Peng Y, Jayasvasti V, Cutler GB Jr., Chrousos GP. Etiology of the differences in corticotropin-releasing hormone-induced ACTH secretion of black and white women. J Clin Endocrinol Metab.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00623-13 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Adrenal Physiology, Pathophysiology, and Molecular Biology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G.B. Cutler	Head	SDE, DEB, NICHD
Others:	K.M. Barnes	Chemist (Tech)	SDE, DEB, NICHD
	A. Chabra	Summer IRTA	SDE, DEB, NICHD
	G.P. Chrousos	Chief, SPE	SPE, DEB, NICHD
	F. Czerwiec	Clinical Associate, NICHD	SDE, DEB, NICHD
	D. Merke	Clinical Associate	SDE, DEB, NICHD
	L. Nieman	Clinical Director, NICHD	URM, DEB, NICHD

COOPERATING UNITS *(if any)*

N. Avila, J. Doppman, Radiology, CC; E. Oldfield, Surgical Neurology Branch, NINDS; K. Yu, K. Amikura, D. Fraker, Surgery Branch, NCI; A. Fojo, S. Bates, Medicine Branch, NCI; K. Kalogeras, Clinical Neuroendocrinology Branch, NIMH; J. Jones, CC, NIH

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Developmental Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	2.6	0.4

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We seek to advance understanding of the mechanisms that cause adrenal androgen secretion by the fetal adrenal zone prenatally and by the definitive adrenal cortex during adrenarche, and to improve the diagnosis and treatment of disorders that cause excess adrenal androgen or glucocorticoid secretion, such as premature adrenarche, congenital adrenal hyperplasia, adrenal neoplasms, idiopathic hirsutism, polycystic ovary syndrome, and Cushing syndrome. We also seek to clarify the pathophysiology of primary adrenal insufficiency (Addison's disease) and secondary adrenal insufficiency and to improve the treatment of these conditions.

Children with congenital adrenal hyperplasia are being enrolled into a study to test the hypothesis that growth can be normalized, and the side effects of supraphysiologic glucocorticoid treatment avoided, by a regimen of antiandrogen, aromatase inhibitor, and reduced hydrocortisone dose. The molecular basis of nonclassical 21-hydroxylase deficiency, 11-hydroxylase deficiency, and adrenal hyperplasia congenita is under investigation. Additionally, the potential to cure the 21-hydroxylase deficiency form of congenital adrenal hyperplasia by gene therapy is being explored in a newly recognized animal model of this disorder.

Patients with Cushing syndrome or a pseudo-Cushing state are being studied by several new diagnostic methods to determine the relative diagnostic efficiency of these new methods compared to the old. Oncogene expression in corticotropinomas is also under study in an attempt to elucidate the molecular basis of Cushing disease.

Others:

T. Tajima	Visiting Fellow	SDE, DEB, NICHD
J. Yanovski	Adjunct Scientist	SDE, DEB, NICHD
K. Oerter-Klein	Special Volunteer	SDE, DEB, NICHD

Project Description

Objectives:

This project seeks to increase our understanding of adrenal physiology, pathophysiology, and molecular biology. The goals of the project are the study of fetal adrenal development, adrenarche, and disorders of adrenal function. The principal disorders under study are congenital adrenal hyperplasia, Cushing syndrome, and pseudo-Cushing states.

Methods Employed:

1) Fetal adrenal development - adrenal gland histology and steroid radioimmunoassay in newborn marmosets treated with substances that have been postulated to be trophic for the fetal adrenal zone.

2) Congenital adrenal hyperplasia - steroid radioimmunoassay, evaluation of new treatments such as LHRH analog, antiandrogen (flutamide) and aromatase inhibitor (testolactone); adrenal transplantation and transgenic techniques, using a newly recognized mouse model of 21-hydroxylase deficiency, to test the hypothesis that the enzyme deficiency can be cured through these approaches.

3) Cushing syndrome and pseudo-Cushing states - evaluation of corticotropin-releasing hormone, overnight dexamethasone, overnight metyrapone, and combined dexamethasone/petrosal sinus catheterization as new approaches for the differential diagnosis of Cushing syndrome; oCRH test to predict recurrence of Cushing syndrome after successful microadenomectomy; oncogene expression in corticotropinomas (extraction of tumor mRNA and identification and analysis of suspected oncogenes by RT-PCR).

4) Adrenal insufficiency - Steroid and peptide radioimmunoassay to study feedback regulation of adrenocorticotropin (ACTH) by cortisol.

Progress:

1. Congenital adrenal hyperplasia

a. Glucocorticoid dose schedule in the treatment of CAH

Previous studies suggested that it might be possible to develop a once-daily hydrocortisone schedule for the treatment of CAH. To test this hypothesis, once-daily and twice-daily schedules were compared in a double-blind, randomized cross-over trial of 2 years' duration (one year on each arm). Total hydrocortisone dose was permitted to be adjusted throughout both arms, as would be the case in clinical practice, and thus the total daily hydrocortisone dosage for each arm was one outcome variable of the study. Despite a 40% increase of hydrocortisone during the once-daily regimen, both the clinical and biochemical measures of control of CAH deteriorated significantly compared to the twice-daily regimen. We concluded that CAH cannot be controlled as effectively with a once-daily regimen, even with a substantial increase in total daily hydrocortisone dose. The results of this study are now being prepared for publication.

b. Supplemental salt administration in patients with CAH

Hypovolemia due to impaired mineralocorticoid synthesis is a major stimulus to ACTH secretion in CAH. Although administration of fludrocortisone should in theory correct mineralocorticoid deficiency, the appropriate dose of fludrocortisone depends upon dietary salt intake and varies accordingly. Thus, patients on a fixed dose of fludrocortisone may have too much mineralocorticoid on some days and too little on others, due to fluctuations in dietary salt. We therefore initiated a randomized double-blind clinical trial to test the hypothesis that supplemental salt administration will reduce day-to-day fluctuation in ACTH secretion, and thus improve control of androgen secretion, by eliminating fluctuations in the hypovolemic stimulus to ACTH release. The results, which indicate effects that are too small to be of clinical importance, are currently being prepared for publication.

c. Treatment of CAH with a new regimen of flutamide, testolactone, and reduced hydrocortisone dose

The successful use of antiandrogen in familial male precocious puberty, combined with inhibition of androgen-to-estrogen conversion, has led us to initiate a similar approach in congenital adrenal hyperplasia. The protocol employs flutamide as the antiandrogen and testolactone as the inhibitor of androgen to estrogen conversion. The rationale for this approach is that androgen excess is an inevitable outcome of physiologic hydrocortisone dosage in CAH. Blockade of excess androgen offers the prospect of normal growth, whereas the conventional approach of suppressing excess androgen through supraphysiologic hydrocortisone dosage does not, because of the growth-inhibiting actions of glucocorticoid.

Preliminary results of this new approach are currently in press in the October, 1996 issue of the Journal of Clinical Endocrinology and Metabolism. Compared to conventional treatment, the regimen of flutamide, testolactone, and reduced hydrocortisone dose produced an increase in plasma 17-hydroxyprogesterone levels and a significant decline in urine cortisol, linear growth rate, weight velocity, and bone maturation. No important adverse effects were observed. We conclude that the regimen of flutamide, testolactone, and reduced hydrocortisone dose improves the short-term control of growth and bone maturation in children with congenital adrenal hyperplasia. Long-term studies have been initiated under protocol 96-CH-0033 to determine whether this approach can normalize these children's growth and development.

d. Testicular adrenal rest tissue in boys with congenital adrenal hyperplasia

One of the most troublesome complications of CAH in boys is the development of testicular adrenal rest tumors. This complication is thought to arise because of increased ACTH levels acting on adrenal rest cells within the testis, an occurrence which has been described in approximately 10% of normal subjects. Thus, those boys with CAH who have adrenal rest cells within the testis are presumed to be at risk for this complication, whereas those who do not have such cells present are presumed not to be at risk.

Although at present there is no way to determine which boys are at risk, it would be desirable to detect these lesions as early as possible so that attempts could be made to assure the optimal degree of ACTH suppression. To improve detection, Avila and his colleagues have performed a detailed analysis of the gray scale and color Doppler ultrasound features of the testicular adrenal rest tissues in 8 boys with this rare condition, which was recently published in Radiology. More recently, we have begun a new study to compare state-of-the-art ultrasound examination with magnetic resonance imaging. Through earlier detection, we hope to develop improved methods to prevent the progression of this complication.

e. Molecular basis of asymptomatic 21-hydroxylase deficiency detected by newborn screening

Although newborn screening for CAH was established to prevent death from salt-losing crisis, these screening programs have also detected asymptomatic cases who do not have salt loss or virilization of the external genitalia (in affected girls). We have performed molecular analysis of 4 such patients from 3 families. Affected children from 2 families had compound heterozygous mutations that included one allele known to be associated with the nonclassical phenotype (Pro30Leu in exon1). The affected girl from the third family was a compound heterozygote for severe mutations normally associated with the salt-losing phenotype. The absence of the expected phenotype in this patient (which is a phenomenon that has been observed by other investigators) implies the existence of factors that can dramatically alter the phenotypic expression of this defect. Determining the nature of these factors could lead to new strategies for treatment. Additionally, improved understanding of the factors that modify phenotype is essential for the accurate use of genotype for prenatal diagnosis and therapy.

2. Cushing syndrome

a. Physiology, pathophysiology, pathogenesis

A longstanding, unresolved question is whether the ACTH-secreting microadenomas in Cushing disease arise through excessive trophic stimulation of normal corticotroph cells. As one approach to this question, we studied basal and corticotropin-releasing hormone (CRH)-stimulated arginine vasopressin (AVP) levels in the inferior petrosal sinus (IPS) of normal volunteers and of patients with Cushing disease. Normal volunteers had both an AVP gradient between the IPS and periphery and an interpetrosal sinus gradient (ISG) (right-to-left or left-to-right gradient) that co-lateralized with the ACTH ISG. This study provides the first direct evidence for the concept of brain lateralization with respect to neuroendocrine secretion. The data are consistent with the hypothesis that CRH-induced AVP release may enhance CRH-mediated ACTH release by the corticotroph, which would represent a novel neuroendocrine mechanism that might be involved in the pathogenesis of Cushing disease.

To explore the potential role of hypothalamic AVP secretion in Cushing disease, we next examined the basal and CRH-stimulated AVP levels in the IPS of patients with Cushing disease. Like the normal volunteers, the patients with Cushing disease had ISG of AVP that co-lateralized with the ACTH ISG. Quantitatively, the CRH-stimulated AVP level of the dominant IPS was 6-8 fold higher in the patients with Cushing disease than in normal volunteers. These findings raise the possibility that high AVP either contributes to corticotroph tumorigenesis, is a consequence of the tumor, or is a result of hypercortisolism. Further studies will be needed to determine the role AVP plays in the etiology of CD.

The CRH test has become an important probe of endogenous CRH secretion in depression and other psychiatric disorders, and an important tool in the differential diagnosis of hypercortisolism. Accordingly, the discovery by Yanovski and coworkers that normal African American subjects secrete approximately twice as much ACTH in response to CRH as caucasian subjects has potentially important pathophysiologic and diagnostic implications. Therefore, studies were undertaken to clarify the mechanism for this observation. There were no racial differences in baseline or CRH-stimulated levels of intact ACTH or cortisol, and no racial differences in adrenal responsiveness to different doses of ACTH. However, after CRH stimulation the plasma of black subjects contained large quantities of degraded yet immunoreactive ACTH molecules (as assessed by HPLC fractionation) that were not observed in the plasma of white subjects. This observation suggests either different processing of CRH-stimulated ACTH molecules prior to secretion, or different patterns of ACTH degradation after secretion. Thus, race is a potentially important variable in clinical studies of the immunoreactive ACTH response to CRH, and clinical tests based on the ACTH response to CRH need to take this racial difference into account.

b. Differential diagnosis

Despite recent advances in transsphenoidal surgery for Cushing disease, the remission rate for all patients undergoing transsphenoidal surgery has remained well below 100 percent. The causes of surgical failure include errors of diagnosis, including the failure to distinguish an ectopic from a pituitary source of ACTH and the failure to distinguish Cushing disease from pseudo-Cushing states, and inability of the surgeon to locate pituitary microadenomas during surgery.

A second problem in differential diagnosis involves the patient with hypercortisolism and a unilateral adrenal mass. In this setting the potential diagnoses range from bilateral ACTH-dependent disorders (pituitary Cushing's disease with macronodular adrenal hyperplasia or incidentaloma) to unilateral or bilateral ACTH-independent disorders (unilateral adrenal adenoma or primary pigmented nodular adrenocortical disease [PPNAD] with a dominant unilateral nodule). A third diagnostic issue involves the patient with factitious Cushing syndrome.

We have recently made the following contributions in the area of differential diagnosis:

1) New criteria have been developed for the single-dose, overnight metyrapone test. Based on a pilot study of this simplified test, the diagnostic efficiency is nearly identical to that of the 2-day standard metyrapone regimen that we described in the Annals of Internal Medicine in 1994.

2) A novel combined dexamethasone/inferior petrosal sinus sampling (IPSS) test appears to improve the discrimination between pituitary Cushing disease and ectopic ACTH syndrome in patients with mild or intermittent hypercortisolism and in patients who have had recent medical treatment for hypercortisolism. Moreover, the absolute level of the CRH-stimulated plasma ACTH in the petrosal sinuses appears to distinguish patients with a pseudo-Cushing state from patients with Cushing disease.

3) The iodocholesterol scan has been shown to be useful in planning adrenal surgery for patients with Cushing syndrome, particularly in distinguishing patients who require bilateral adrenalectomy from those in whom unilateral adrenalectomy is indicated.

4) Clinical and laboratory approaches to the diagnosis of factitious Cushing syndrome have been developed.

1) New diagnostic criteria for the single-dose overnight metyrapone test

A study involving 63 patients has been conducted to determine whether this simplified test, requiring 12 hours rather than the 48 hours for the standard test, is as effective diagnostically as the standard test. Stimulation of 11-deoxycortisol > 220-fold, or a decrease of plasma cortisol during metyrapone of < 40%, yielded a specificity of 100% and a sensitivity of 65% for the diagnosis of Cushing disease. The sensitivity of 65% was nearly identical to that obtained with the standard test in the same patients (67%). We conclude that the single-dose test, which avoids the need for timed urine collections, has nearly identical diagnostic efficiency as the standard test. Moreover, combining the results from the two tests increased sensitivity to 84%, which was significantly greater than with either test alone ($p < 0.02$). This illustrates the clinical value of performing additional tests when a single test yields negative or equivocal results. The results of this study have been accepted for publication in Clinical Endocrinology. We ultimately envision that this test could be combined with two other noninvasive, single-dose tests (8-mg overnight dexamethasone test and the ovine corticotropin-releasing hormone test) to permit accurate non-invasive diagnosis of Cushing disease in nearly all subjects.

2) Combined dexamethasone/inferior petrosal sinus sampling (DEX/IPSS) test

One of the pitfalls of the IPSS procedure to distinguish ectopic from pituitary sources of ACTH is the patient with an ectopic source of ACTH who has had recent adrenal blockade or who has mild or intermittent hypercortisolism. The normal pituitary corticotroph cells in such patients may be unsuppressed, giving rise to a petrosal sinus-to-peripheral ACTH gradient that mimics the gradient seen in Cushing disease. To overcome this problem, we hypothesized that the administration of dexamethasone (8 mg per day for 2 days) prior to IPSS would fully suppress normal, but not adenomatous, corticotroph cells, leading to improved diagnosis. The results indicate that this is indeed the case, and that the DEX/IPSS test will improve diagnostic accuracy in this setting. These findings are currently be prepared for publication.

3) Iodocholesterol scan for definitive differentiation of unilateral from bilateral disorders in patients undergoing adrenalectomy for Cushing's syndrome

In a collaborative study (with the Surgery Branch of the National Cancer Institute) in 12 patients who were proven by unilateral adrenalectomy to have hypercortisolism due to unilateral disease (11 with adrenal adenoma, 1 with ACTH-independent nodular cortical hyperplasia), the iodocholesterol scan was 100% accurate in indicating the unilateral source of the hypercortisolism, whereas CT and MR scan suggested a bilateral source in 2 of the cases because of nonfunctioning cortical nodules in the contralateral adrenal glands. Among 11 patients with bilateral adrenal hyperfunction, the iodocholesterol scan was 91% sensitive in indicating bilateral disease, with one false-negative result due to bilateral nonvisualization of the adrenals. Thus, the iodocholesterol scan is highly effective in confirming the unilaterality of adrenal adenoma versus the bilaterality of ACTH-dependent macronodular adrenal hyperplasia (with a dominant unilateral nodule) or of ACTH-independent PPNAD.

4) Diagnosis of factitious Cushing syndrome

To develop a more efficient strategy for the recognition and diagnosis of patients with factitious Cushing syndrome, we reviewed the clinical and laboratory features of 6 factitious patients who were identified among more than 800 patients with hypercortisolism referred to the NIH. Like other cases of Munchausen syndrome, the patients with factitious Cushing syndrome had a high incidence of family or work contact with the medical profession, of multiple unrelated surgical procedures, and of psychiatric illness. The most useful laboratory test was high-pressure liquid chromatographic (HPLC) analysis of urine steroids, which revealed synthetic glucocorticoids in 4 of 6 patients. Also helpful was hormonal evidence of adrenal suppression or rapid alternation of adrenal function between very high and normal or low values. Thus, the study suggests clinical features that should arouse concern of factitious disease and provides an efficient laboratory approach to confirm the diagnosis.

c. Treatment

1) Recovery from osteoporosis after cure of Cushing syndrome

Osteoporosis is a well-recognized complication of Cushing syndrome, although relatively little is known about the natural history of this complication in treated patients. To gain further information about the potential of osteoporotic bone in Cushing syndrome to recover normal bone density after cure of hypercortisolism, patients with Cushing syndrome are undergoing bone density measurements by dual energy x-ray absorptiometry (DEXA) before and after successful treatment. If patients fail to show recovery of bone density within several years after cure, we plan to initiate a therapeutic trial to determine whether bone density can be improved by any of the treatments employed in the treatment of idiopathic or postmenopausal osteoporosis. Data collection for this study will be completed within the next year.

2) Mechanism of hypogonadism in Cushing syndrome and effect of successful treatment

Decreased plasma testosterone, impotence, and decreased libido are common in men with Cushing syndrome. Hypogonadism also occurs commonly in women with Cushing syndrome, but is more difficult to study because of the need to control for the stage of the menstrual cycle. To gain further insight into the mechanism of hypogonadism in Cushing syndrome, spontaneous gonadotropin secretion and the gonadotropin response to administration of gonadotropin-releasing hormone have been studied before and after successful treatment. The results of this study are currently being analyzed.

3) Drug treatment of ACTH-secreting tumors in Cushing disease and in Nelson syndrome

Following bilateral adrenalectomy for Cushing disease, the pituitary tumors sometimes enlarge and secrete markedly increased amounts of ACTH (Nelson syndrome). Although 3 drugs have had limited effectiveness in suppressing the function of these tumors (cyproheptadine, bromocriptine, and valproic acid), none has achieved widespread clinical acceptance because of a low response rate. Since these 3 drugs are believed to act through different mechanisms, we conducted a study to determine whether the simultaneous use of these agents might be additive or synergistic in suppressing ACTH secretion in persistent Cushing disease after unsuccessful transsphenoidal surgery and in Nelson syndrome. With the single-dose study design that was employed, only bromocriptine and the combination of all three agents achieved statistically significant suppression of plasma ACTH. Addition of cyproheptadine and valproic acid did not have any further ACTH-suppressing effect. The results of this study are currently under review at the Journal of Clinical Endocrinology and Metabolism.

4) Surgical treatment of ACTH-producing pancreatic islet cell tumors

Ectopic ACTH-producing pancreatic islet cell tumors usually have a progressive clinical course and fatal outcome. Since the benefit of aggressive surgical resection of primary and metastatic lesions has not been established, the outcome of 12 cases treated at the NIH was reviewed through a collaborative study (with the Surgery Branch, NCI). Despite aggressive surgery, including 3 patients who underwent distal pancreatectomy and hepatic resection, none of the patients had biochemical evidence of cure. Bilateral adrenalectomy, however, successfully controlled Cushing's syndrome in the 8 patients in whom it was performed. The results of this study do not support a role for aggressive resection of the primary tumor, but do confirm that palliative bilateral adrenalectomy is justified.

5) Function and regulation of the multi-drug resistance gene product, p-glycoprotein, in human adrenocortical cancer cell lines

One hypothesis for the primary resistance of adrenocortical cancer to chemotherapy is its high baseline expression of p-glycoprotein, an ATP-dependent membrane protein that pumps polycyclic chemotherapy compounds (such as adriamycin, etoposide, and VP-16) out of cells and thereby reduces their cytotoxicity. To explore further the role of p-glycoprotein in cultured adrenocortical cancer cells, we examined their ability to transport, and to be inhibited by, a large number of endogenous and synthetic steroid molecules. Most steroid molecules either inhibited p-glycoprotein-mediated transport or were themselves transported by p-glycoprotein. In general, the more polar steroids, like cortisol, were transported by p-glycoprotein, whereas the less polar steroids, like progesterone, blocked pump activity but were not themselves transported. Lastly, the study showed that the effect of steroids on p-glycoprotein activity was modulated by the level of phosphorylation of p-glycoprotein. These observations suggest the hypothesis that p-glycoprotein may play a physiological role in the transport of polar steroids across adrenal cell membranes. The also suggest that strategies to inhibit the p-glycoprotein activity of adrenal cancer cells may increase their sensitivity to chemotherapy.

Significance:

1) Fetal adrenal zone and adrenarche. Adrenal androgens from fetal adrenal are the major precursors for placental estrogen synthesis during pregnancy, which plays an important role in preparing the breast for lactation and in inducing uterine oxytocin receptors. Postnatally, adrenal androgens play an important pathogenetic role in hormone-dependent cancers such as those of the breast and prostate, and in several endocrine disorders such as congenital adrenal hyperplasia, premature adrenarche, hirsutism, and Cushing syndrome. Increased understanding of the mechanisms regulating adrenal androgens may lead to new methods of controlling adrenal androgen secretion in these disorders.

2) Congenital adrenal hyperplasia (CAH). This is among the most common autosomal recessive disorders in the United States population. The classic form affects approximately 1 in every 13,000 children (about the same as PKU), and has a heterozygote frequency of about 1 in 57. The nonclassical or late-onset form of CAH is even more common. Thus, advances in the treatment of this disorder will benefit the 300 children with classic CAH and the larger number of nonclassical patients born annually in the United States.

3) Hirsutism. Approximately 1 woman per 1000 (a prevalence of nearly 100,000 women in the United States) has hirsutism sufficiently severe to constitute a significant management problem. These women often develop anxieties about their feminine identity that hinder normal social development during the teen-age and adult years. Thus, improved understanding of the pathogenesis of this disorder, and improved treatment methods, could enhance the well-being of a large number of women.

4) Cushing disease. This disorder is estimated to affect 600 to 2000 new cases per year in the United States. When described by Cushing in 1932, it was a devastating, uniformly fatal illness that often struck in the prime of life. Recent advances have greatly improved our ability to diagnose accurately and to treat effectively this potentially lethal disease.

5) Adrenal insufficiency. This potentially fatal disorder continues to pose a therapeutic challenge because of the morbidity associated with undertreatment or overtreatment. Improved methods of treatment are needed to restore these patients to full health.

Proposed Course:

1) The hypothesis that adrenarche results from a proopiomelanocortin (POMC)-derived peptide will be tested by comparing the adrenal androgen response to corticotropin-releasing hormone (CRH), which stimulates POMC, to that of ACTH, which suppresses POMC. This study will be carried out in patients with hypothalamic adrenal insufficiency.

2) Congenital adrenal hyperplasia. Future studies will address the following areas:

a) The effect on growth rate, bone maturation, predicted adult height, and final adult height of a new regimen consisting of antiandrogen (flutamide), aromatase inhibitor (testolactone), reduced hydrocortisone dose, and fludrocortisone versus a conventional hydrocortisone/fludrocortisone regimen. Other proposed new clinical projects include the use of a CRH antagonist or a delayed release hydrocortisone formulation to block the early morning increase in ACTH secretion.

b) The effect, in a recently discovered strain of mice with 21-hydroxylase deficiency, of this disorder on the development of the hypothalamic CRH neuron, as determined by immunohistochemistry and in situ hybridization methods.

c) Use of the 21-hydroxylase-deficient mouse to develop approaches to correct the 21-hydroxylase deficiency by gene therapy approaches.

Testing of new therapeutic strategies would benefit from the recruitment of a birth cohort of CAH patients whose potential adult height has not been compromised by inadequate treatment during the first 1-2 years of life. To identify such a cohort, we are attempting to interest the nearby state laboratories responsible for newborn screening in setting up a pilot newborn screening program. Effective, inexpensive methods for newborn screening of CAH have been developed and are in use in Italy, Japan, and the states of Washington, Alaska, Illinois, Texas and other states.

3) Cushing syndrome

To improve the usefulness of inferior petrosal sinus sampling (IPSS) in the differential diagnosis of Cushing disease versus ectopic ACTH secretion, the diagnostic efficiency of the new DEX/IPSS test will be analyzed. To decrease the time and cost of evaluation of hypercortisolism, a 1-day metyrapone test, dexamethasone test, and oCRH test will be evaluated prospectively. The goal of a single outpatient test with improved diagnostic efficiency will be pursued as promising new approaches become available. Other initiatives include efforts to define the natural history of the osteoporosis and hypogonadism of Cushing disease before and after successful transsphenoidal microsurgery. If the osteoporosis is not reversible with cure of hypercortisolism, new therapeutic strategies will be developed to attempt to correct the loss of bone density.

To gain insight into the molecular basis for the corticotroph neoplasms that cause Cushing disease, RNA from these neoplasms will be amplified by RT-PCR to detect the presence of potential oncogenes. Our ultimate goal is to gain new insights into the molecular basis for this disorder that can be used to design innovative strategies to eradicate these tumors or to inhibit their growth and excessive hormone secretion.

Protocols:

Animal

92-016	Tajima	Comparison of the density of hypothalamic corticotropin-releasing hormone neurons between normal mice and mice with congenital adrenal hyperplasia

Human

90-CH-194	Yanovski	Inferior petrosal sinus sampling for the determination of adrenocorticotropic hormone (ACTH) concentration in normal volunteers and in patients with disorders of the hypothalamic-pituitary-adrenal axis

This study is currently testing the hypothesis that the 2-day, 8-mg dexamethasone test administered before inferior petrosal sinus sampling (Dex/IPSS test) will suppress normal corticotroph function during the IPSS procedure but will permit continued ACTH secretion from ACTH-secreting pituitary microadenomas causing Cushing disease. If correct, this hypothesis implies that the Dex/IPSS test will extend the diagnostic usefulness of IPSS to patients with intermittent Cushing syndrome, mild Cushing syndrome, or prior adrenal blockade. These are settings in which the conventional IPSS procedure may yield misleading results because central-to-peripheral ACTH gradients arising from normal corticotroph cells may be mistaken for an ACTH-secreting microadenoma. Thus, an erroneous diagnosis of pituitary Cushing disease may be made in the patient with ectopic ACTH syndrome or with an unrecognized pseudo-Cushing state.

The study employs a randomized, open design in which the patient receives 2 IPSS procedures, one with and one without prior 8 mg per day dexamethasone administration for 2 days. The diagnostic performance of the conventional and modified IPSS procedure will then be compared against the "gold standard" diagnosis determined at follow-up (the "gold standard" is surgical cure or pathologic diagnosis for Cushing syndrome and remission or prolonged lack of progression for pseudo-Cushing states).

Preliminary results suggest that the Dex/IPSS procedure has improved diagnostic accuracy compared to IPSS in settings in which normal corticotroph function may not be suppressed. We conclude that further evaluation of this new test is warranted.

94-CH-0144	Winer, Cutler	Bone mineral density and bone metabolism in patients with untreated and cured endogenous Cushing syndrome

This study tests the hypothesis that patients with osteoporosis owing to Cushing syndrome will recover normal bone density within 3 years of the cure of Cushing syndrome. We further hypothesize that this recovery of bone density will be attributable to an increased rate of bone formation without a corresponding increase in bone resorption. The study employs a longitudinal, observational design in which each subject's pretreatment bone density, bone formation markers, and bone resorption makers are compared by ANOVA for repeated measures with the corresponding values during the 3 years after the cure of Cushing syndrome.

The available pretreatment results indicate low-turnover osteoporosis before treatment of Cushing syndrome, indicating that both the rate of bone formation and of bone resorption are suppressed. Further observation will be required to determine the changes following the cure of Cushing syndrome.

96-CH-0033	Cutler	An open, randomized, long-term clinical trial of flutamide, testolactone and reduced hydrocortisone dose versus convential treatment of children with congenital adrenal hyperplasia

Total accrual approved: 60
Accrual to date: 36
Project start date: 1996
Projected finish date: 2011

This study tests the hypothesis that the regimen of flutamide, testolactone, and reduced hydrocortisone dose will normalize the growth and development of children with congenital adrenal hyperplasia (CAH). The study employs a randomized, open, parallel design comparing the new regimen to conventional therapy throughout the prepubertal period. Once central puberty has begun, both groups will be treated with a conventional hydrocortisone and fluorocortisone regimen until adult height is achieved.

The preliminary results have shown decreased growth velocity, weight velocity, and bone maturation rate during the new regimen despite the decrease in glucocorticoid dose and increase in the adrenal androgen precursor, 17-hydroxyprogesterone, in plasma. Only one child in 26 has failed to tolerate the new regimen, due to GI intolerance. No other adverse effects have been observed. We conclude that the new regimen has short-term effectiveness and deserves longer term study.

Publications:

Avgerinos PC, Nieman LK, Oldfield EH, Cutler GB Jr. A comparison of the overnight and the standard metyrapone test for the differential diagnosis of Cushing syndrome. Clin Endocrinol, in press.

Avila NA, Premkumar A, Shawker TH, Jones JV, Laue L, Cutler GB Jr. Testicular adrenal rest tissue in congenital adrenal hyperplasia: gray scale and color Doppler ultrasound. Radiology 1996;198:99-104.

Barnes KM, Dickstein B, Cutler GB Jr, Fojo AT, Bates SE. The accumulation of steroids in multidrug-resistant cells and steroid antagonism of the P-glycoprotein drug efflux pump can be decreased by the PKC inhibitor, calphostin C. Biochemistry 1996;35:4820-7.

Cizza G, Nieman LK, Doppman JL, Passaro MD, Czerwiec FS, Chrousos GP, Cutler GB Jr. Factitious Cushing syndrome. J Clin Endocrinol Metab, in press.

Cutler GB Jr. Ambiguous genitalia. In: Hurst JW ed. Medicine for the practicing physician, 4th ed. Stamford, Connecticut: Appleton Lange, 1996;617-23.

Cutler GB Jr. Treatment of congenital adrenal hyperplasia: the case for new medical approaches. J Clin Endocrinol Metab, in press.

Czerwiec FS, Cutler GB Jr. Modern approach to corticosteroid replacement. Curr Opinion Endocrinol Diabetes 1996;3:239-46.

Friedman TC, Nieman LK, Yanovski JA, Doppman JL, Cutler GB Jr., Chrousos GP, Kalogeras KT. Inferior petrosal sinus arginine vasopressin concentrations in normal volunteers and patients with Cushing disease. J Clin Endocrinol Metab 1996;81:3068-72.

Kalogeras KT, Nieman LK, Friedman TC, Doppman JL, Cutler GB Jr., Chrousos GP, Wilder RL, Gold PW, Yanovski JA. Inferior petrosal sinus sampling in normal human subjects reveals a unilateral corticotropin-releasing hormone-induced arginine vasopressin release associated with ipsilateral adrenocorticotropin secretion. J Clin Invest, in press.

Klein KO, Cutler GB Jr. The hypothalamus and neuroendocrine disorders. In: Berg BO, ed. Principles of child neurology. New York: McGraw-Hill, 1996;437-57.

Laue L, Cutler GB Jr. The adrenal cortex. In: Bittar EE, Bittar N, eds. Principles of medical biology. Greenwich: JA1 Press, in press.

Laue L, Merke DP, Jones JV, Barnes KM, Hill SV, Cutler GB Jr. A preliminary study of flutamide, testolactone, and reduced hydrocortisone dose in the treatment of congenital adrenal hyperplasia. J Clin Endocrinol Metab, in press.

Nieman LK, Cutler GB Jr. Cushing's syndrome. In: DeGroot LJ, ed. Endocrinology, 3rd ed. Philadelphia: W.B. Saunders, 1995;1741-69.

Sartor O, Cutler GB Jr. Treatment of Cushing's syndrome with the antiglucocorticoid mifepristone (RU486). Clin Obstet Gynecol 1996;39:506-10.

White PC, Pescovitz OH, Cutler GB Jr. Synthesis and metabolism of corticosteroids. In: Becker KL, ed. Principles and practice of endocrinology and metabolism, 2nd ed. New York: J.B. Lippincott, in press.

Yanovski JA, Yanovski SZ, Friedman TC, Loh YP, Jayasvasti V, Cutler GB Jr, Chrousos GP. Etiology of the differences in corticotropin-releasing hormone-induced ACTH secretion of black and white women. J Clin Endocrinol Metab, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00627-07 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Glycoprotein Hormones: Oligosaccharide Structure and Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	D. Blithe	Expert	UG, SME, DEB, NICHD
Others:	M. Nemansky	Visiting Fellow	UG, SME, DEB, NICHD
	E. Moy	Clinical Associate	UG, SME, DEB, NICHD
	C. Lyons	Technician	UG, SME, DEB, NICHD

COOPERATING UNITS *(if any)*

R. Iles, St. Bartholomew's Hospital Medical College, West Smithfield, London, U.K.; V. Reinhold, Boston University School of Medicine, Boston, MA

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Unit of Glycobiology

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of the project is to increase our understanding of the structure-function relationships and glycobiology of the glycan moieties on glycoprotein hormones. Molecules under investigation are, human chorionic gonadotropin (hCG), gonadotropin free α subunits associated with pregnancy or malignancy, and pituitary free α secreted throughout the normal menstrual cycle. We showed that free α stimulated secretion of prolactin and IGFBP-1 from primary cultures of human decidual cells in a dose-dependent manner. This finding indicated that free α is a glycoprotein hormone with functions that are independent of heterodimeric gonadotropins. We showed that free α demonstrates specific binding to endometrial stromal cells and plays a role in recruitment of cells for decidualization. This observation expands the function of the free α to include reproductive physiology associated with the luteal phase of the menstrual cycle and may indicate involvement in implantation.

Carbohydrate modifications, resulting in a variety of branched glycan structures, occur on all glycoproteins prior to secretion. These modifications can affect virtually every aspect of the molecule's behavior, including receptor binding and signal transduction. We have investigated functional aspects of the glycan moieties on free α, demonstrating that specific types of carbohydrate modifications on free α can either prevent or facilitate combination with hCG-β to form intact hormone. We have shown that the glycan moieties on early pregnancy free α function to maintain the molecule in an uncombined form in placental compartments that also contain high amounts of combinable free β-subunit. We have identified differences in the glycan moieties of free α as a function of gestational development; in particular, the glycosylation of free α changes dramatically during the second trimester of pregnancy. Molecules produced in late pregnancy are more highly branched and are more fucosylated than those of early pregnancy. In view of the dramatic changes in glycosylation, it will be important to evaluate their contribution to free α bioactivity.

Project Description:

Objectives:

The overall objective of the project is to further our understanding of the structural elements of glycoprotein hormones and how these elements, particularly the carbohydrate moieties, contribute to the function of these molecules. The molecules under study were, human chorionic gonadotropin (hCG), and a "new hormone", gonadotropin free α subunit.

We have previously described a function for free α subunit in pregnancy, demonstrating that it stimulates secretion of prolactin from primary cultures of human decidual cells. In view of our findings, we proposed that free α is a glycoprotein hormone with activity that is independent of hCG. Although free α reaches its highest maternal serum levels during late pregnancy, much higher concentrations of free α are observed in placental compartments (extraembryonic coelomic fluid and amniotic fluid) in early pregnancy. Thus, free α may have a functional role in early pregnancy, in addition to regulating prolactin secretion in late pregnancy. Free α subunits are also secreted by the pituitary throughout the normal menstrual cycle in nonpregnant individuals. The hypothesis that free α is an independent glycoprotein hormone defines a new area of basic and clinical research on the functions, physiology, and glycobiology of free α molecules.

In view of the dominant role that glycosylation can play in regulating hormonal activity, our goal is to elucidate the structure and function of the carbohydrate moieties on pregnancy-related hormones. Changes in glycosylation of these molecules were examined as a function of gestational development. Knowledge of the glycan structures on hCG and free α is critical for determining the contribution of specific glycans to the functions of these molecules. Also, knowledge of the normal structures of these molecules throughout pregnancy provides information about the regulation of glycosylation during pregnancy and provides a basis for distinguishing aberrant structures in diseases of pregnancy.

Methods Employed:

Studies on hCG and free α molecules that were secreted at different times during pregnancy were performed on urine samples collected at weekly intervals throughout five individual pregnancies. Amounts of hCG, free α, hCG-β subunit, human prolactin, and IGFBP-1 were assessed by radioimmunoassay using polyclonal or monoclonal antisera. In some cases, purification of hCG from free α was not desirable, therefore, we used monoclonal antibodies in a radioimmunoassay that recognized either intact hCG or free α with minimal cross reactivity. For structural characterization of hCG and free α, the hormones were purified by gel filtration and immunoaffinity chromatography. Carbohydrate structure was determined by lectin affinity chromatography using lectins with a variety of carbohydrate specificities and by mass spectrometry of permethylated glycans. Carbohydrate composition was determined after acid hydrolysis of purified material. Monosaccharides were separated by high pH-anion exchange HPLC and were quantified by pulsed amperometric detection.

For the free α bioassay and for binding studies, primary cell cultures of endometrial stromal cells were established from endometrial biopsies obtained during various times of the menstrual cycle. A variety of standard biochemical methods were also employed in these studies, including enzymatic digestion, density gradient centrifugation, SDS-polyacrylamide gel electrophoresis, immunoblotting, etc.

Major Findings:

We have postulated a novel function for free α in pregnancy and in endometrial differentiation during the normal menstrual cycle. We had previously shown that the free α molecule purified from pregnancy urine stimulates secretion of prolactin from primary cultures of human decidual cells isolated from term pregnancies. The concentration of free α that stimulated prolactin was well within the physiologic maternal serum free α levels during pregnancy. Thus, placental free α molecules appear to have a functional role in pregnancy of stimulating decidual prolactin secretion, thereby identifying free α as an independent glycoprotein hormone.

We have now extended our findings to show that free α is likely to play an important role in the normal menstrual cycle and perhaps in very early pregnancy. In addition to the fact that free α is a major placental product during pregnancy, free α molecules are also secreted by the pituitary throughout the normal menstrual cycle in a pulsatile fashion that is regulated by GnRH. Using cells obtained by endometrial biopsy, we have found that free α can stimulate endometrial stromal cells to undergo the differentiation process of decidualization. Decidualized endometrium is characterized by morphologic changes and by secretion of prolactin and IGFBP-1, and the process of decidualization is crucial for development of a uterine lining that is receptive for implantation. Addition of free α in a dose range that is within normal physiologic levels of the menstrual cycle results in morphologic changes associated with decidualization with concomitant stimulation of prolactin and IGFBP-1 secretion. The effects of free α are dramatically enhanced by simultaneous addition of progesterone, resulting in a synergistic response of the combined hormones compared to the additive effects of either hormone alone. These findings demonstrate that free α is likely to be playing a role in the preparation of the uterus for receiving the developing embryo. This novel concept may have important implications regarding infertility, recurrent spontaneous abortion and assisted reproduction.

We have recently demonstrated specific binding of the free α subunit to cultured endometrial stromal cells obtained from endometrial biopsy. These results indicate that a receptor for free α subunit is likely to be present on a population of endometrial stromal cells and that the observed bioactivity of free α is a receptor-mediated process.

We have shown previously that modifications to the N-linked glycans of free α occur during glycoprocessing of the molecule resulting in branched glycan structures that can prevent free α from combining with hCGβ-subunit. The glycosylation on free α protects the populations of free α and β subunits that coexist in placental compartments in early pregnancy. However, the glycosylation of hCG and free α had been assumed to be unchanged throughout pregnancy. We have shown that their glycosylation does change as gestation advances. Particularly on free α, and to a lesser extent on hCG, the type of branching and the extent of fucosylation of the glycans was increased significantly in late pregnancy compared with that of early pregnancy. The glycosylation patterns between individual healthy pregnancies were remarkably similar to one another when the same period of gestation was compared, and all of the individuals displayed an increase to more highly branched and more highly fucosylated glycans in late pregnancy. It is important to note that similar changes in glycosylation are also observed after malignant transformation, and that alterations in the glycosylation of hCG and α-fetoprotein have been suggested as potential diagnostic markers for malignancy. However, normative patterns for hCG and free α glycosylation throughout uncomplicated pregnancies have not been established. Since there are extensive parallels between malignant transformation and embryonic development, it is crucial to obtain these normative data in order to establish aberrant glycosylation profiles in disease. We have examined the time points between week 12 and week 28, previously designated as early and late pregnancy, respectively, to determine if the changes in glycosylation were gradual throughout gestation, or occurred suddenly at a specific point in gestational development. In each of the five individual pregnancies we have determined that the bulk of the change occurs between weeks 14 and 16. After reaching the glycosylation pattern achieved by week 18, the profile remains constant for the remainder of the pregnancy. It is likely that the changes in the glycosylation patterns reflect alterations in key glycoprocessing enzymes that are under developmental regulation. We have performed detailed carbohydrate analysis on the glycans of early and late free α molecules using mass spectrometry analysis of permethylated glycans. The analysis indicated changes in the extent of fucosylation and type of branching of the glycans on free α. The results suggest that during early second trimester of pregnancy, there is a marked increase in the placental expression of two glycosyltransferases (N-acetylglucosaminyl transferase V and α1-6 fucosyl transferase) that are responsible for the addition of specific sugars.

Significance to Biomedical Research and the Program of the Institute:

In spite of the copious production of gonadotropin free α subunit in pregnancy, no biological role had been established for it. We have now identified two important activities of free α. Our most recent experiments indicate that free α can play a role in decidualization of endometrial cells in the normal menstrual cycle. These results expand our previous observation that free α can stimulate prolactin secretion from terminally differentiated decidual cells to suggest that free α functions in the preparation of the uterine lining for implantation as well as the maintenance of the established pregnancy. We have demonstrated specific binding of the free α molecule suggesting that the bioactivity occurs through a receptor-mediated event. Our findings establish free α as a glycoprotein hormone with functions that occur during the normal menstrual cycle as well as during very early pregnancy when free α is being secreted by trophoblast cells of the developing embryo. This novel concept will require expansion of the scope of endocrinological investigations to include potential contributions from free α, in addition to those of the heterodimeric members of the gonadotropin family. Elucidation of the functions of free α may have important implications for certain cases of infertility, luteal phase defects, or recurrent miscarriage. Furthermore, a closer monitoring of the free α subunit levels in procedures used for assisted reproduction may be necessary. It is conceivable that free α may have a role in implantation such that too much or too little free α could be undesirable.

We previously had compared the carbohydrate structures on the free α molecule in pregnancy with those on the hCG-α subunit isolated after dissociation of intact hCG. Although the two α molecules contain the same polypeptide structure, we showed that they have distinctly different carbohydrate moieties and that these carbohydrate structures could either prohibit or facilitate combination with hCG-β subunit to form intact hCG. In normal pregnancy, the vast majority of α polypeptides that are synthesized become free α molecules.

The glycosylation of these molecules had been assumed to be unchanged throughout pregnancy, however, we have now shown that glycosylation changes dramatically as gestation advances. In late pregnancy, the amount and type of branching as well as the extent fucosylation of the glycans on free α, and to a lesser extent on hCG, was increased significantly compared to that of early pregnancy. The dramatic rise in fucosylation of free α as gestation advances is not reflected as a general increase in fucosylation of secreted glycoproteins in pregnancy and thus likely reflects specific changes in the trophoblast cells that produce free α molecules. As yet, we have not observed any exceptions to the glycosylation changes; all five pregnancies exhibited similar changes in glycosylation of late pregnancy free α. Studies on free α glycosylation may provide important clues about the regulation of the trophoblast glycosyl transferases. Having demonstrated that there are developmentally associated changes in the glycosylation of free α, we have narrowed the gestational window of these changes to weeks 14-16 of pregnancy.

It is important to note that similar changes in glycosylation branching and fucosylation are also observed after malignant transformation, and alterations in glycosylation of hCG and α-fetoprotein have been suggested as potential diagnostic markers for malignancy. However, normative patterns for glycosylation of these molecules throughout uncomplicated pregnancies had not been established. Since there are extensive parallels between malignant transformation and embryonic development, it was crucial to obtain these normative data in order to establish aberrant glycosylation profiles in disease.

Proposed Research:

We have described preliminary findings indicating that free α is likely to be a glycoprotein hormone with activity that is independent of hCG. This novel concept will require expansion of the scope of endocrinological investigations to include potential contributions from free α, in addition to those of the other members of the gonadotropin family. The goal of future research will be to elucidate the function and glycobiology of free α and to identify the receptor-mediated pathway of its bioactivity. Having demonstrated that there are dramatic structural differences on free α between early and late pregnancy, it will be important to examine how these structures contribute to the newly observed bioactivity of free α.

Protocols:

93-CH-0075	Blithe	The role of gonadotropin free α subunit in the regulation of decidual cell function.
B-93-009(NNMC)	Nash	The role of glycoprotein hormone alpha subunit in the regulation of human decidual function in pregnancy.

Publications:

Moy E, Kimzey LM, Nelson LM, Blithe DL. Glycoprotein hormone α-subunit functions synergistically with progesterone to stimulate differentiation of cultured human endometrial stromal cells to decidualized cells: a novel role for free α-subunit in reproduction. Endocrinology 1996;137,1332-9.

Patents:

Blithe DL, Wehmann RE, Nisula BC. U.S. Serial No. 07-292,985: Purification of human chorionic gonadotropin β-core molecule and preparation of antibodies with specificity for same, patent approved. Product licensed to Ciba Corning Diagnostics Corporation.

Blithe DL, Wehmann RE, Nisula BC. U.S. Serial No. 08-448,079; Purification of human chorionic gonadotropin β-core molecule and preparation of antibodies with specificity for same. Continuation-in-part of U.S. Serial No. 07-292,985. Foreign filing license granted. Patent pending.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00628-07 DEB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biological roles and mechanisms of action of insulin-like growth factors (IGFs)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	C.A. Bondy	Senior Clinical Investigator	SGM, DEB, NICHD
Others:	Jian Zhou	Visiting Associate	SGM, DEB, NICHD
	Rick Reinhardt	Senior Staff Fellow	SGM, DEB, NICHD
	Jie Wang	Biologist	SGM, DEB, NICHD
	Oluyemisi Adesanya	Clinical Associate	SGM, DEB, NICHD
	Sui Ng	IRTA Fellow	SGM, DEB, NICHD
	Serge Simpson	Pre-IRTA Fellow	SGM, DEB, NICHD
	George T. Joncas	Pre-IRTA Fellow	SGM, DEB, NICHD

COOPERATING UNITS *(if any)*

Genentech, San Francisco, CA (L. Powell-Braxton); Diabetes Branch, NIDDK (D. LeRoith, I. Simpson); Department of Genetics, Columbia (A. Efstratidis)

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Growth and Metabolism

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.35	4.85	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This Section carries out research on the mechanisms of growth regulation, focusing on the actions of insulin-like growth factors -I and -II (IGFs-I and -II). These growth factors have major roles in development as proven by the fact that targeted deletion of IGF-I and IGF-II gene expression results in severe dwarfism and, in the case of IGF-I, infertility in both sexes--although how IGF deletion causes these defects is not yet understood. The Section investigates the molecular mechanisms of IGF action in regulating growth and reproduction by utilizing these IGF 'knockout' mice models.

Over the past year the signal transduction pathways and biological functions of IGFs in the brain have been elucidated. Analysis of IGF-I null brains has revealed major disruptions of cerebral metabolism, including a decrease in neuronal hexokinase enzyme activity and protein expression and a decrease in glucose uptake which is restored by exogenous IGF-I. Pyruvate kinase, neuron-specific enolase, malate dehydrogenase and creatine kinase are also significantly altered in IGF-I null brains, demonstrating that absence of IGF-I results in major disruption of cerebral metabolic pathways and supporting the hypothesis that endogenous IGF-I acts as the brain's insulin. Other studies in the IGF-I null mice have shown that the S-phase of the cell-cycle appears to be prolonged in the knockouts, providing novel insight as to the mechanism of IGF-I's growth promoting effects.

Studies on the role of the IGF system in reproduction have made substantial progress. Studies in the IGF-I knockout mouse have demonstrated that contrary to current views, IGF-I is not the mediator of estrogen's growth-promoting effects on the uterus, but rather is necessary for estrogen production. Other studies suggest that IGF-II deletion causes dwarfism primarily by attenuation of placental growth rather than by affecting the fetus directly.

Period 10/95-9/96 **#HD-00628-** 07 **DEB**

Title: Biological roles and mechanisms of action of insulin-like growth factors (IGFs).

PI:	CA Bondy	SI,	DEB, NICHD
Others:	Jian Zhou	Visiting Assoc	
	Rick Reinhardt	Senior Staff Fellow	
	Jie Wang	Biologist	
	Oluyemisi Adesanya	CA	
	Sui Ng	IRTA	
	Serge Simpson	Pre-IRTA	
	George T. Joncas	Pre-IRTA	

Cooperating units:
Lynn Powell-Braxton, PhD, Genentech, San Fransico
Diabetes Branch, NIDDK (D. LeRoith and I. Simpson)
Arg Efstratidis, Dept of Genetics, Columbia.
DEB
Growth & Metabolism Section
NICHD
Total person years: 6.35 (Prof- 4.85; Other 1.5)
(b) human tissues

Summary:

This Section carries out research on the mechanisms of growth regulation, focusing on the actions of insulin-like growth factors -I and -II (IGFs-I and -II). These growth factors have major roles in development as proven by the fact that targeted deletion of IGF-I and IGF-II gene expression results in severe dwarfism and, in the case of IGF-I, infertility in both sexes—although how IGF deletion causes these defects is not yet understood. The Section investigates the molecular mechanisms of IGF action in regulating growth and reproduction by utilizing these IGF 'knockout' mice models.

Over the past year the signal transduction pathways and biological functions of IGFs in the brain have been elucidated. Analysis of IGF-I null brains has revealed major disruptions of cerebral metabolism, including a decrease in neuronal hexokinase enzyme activity and protein expression and a decrease in glucose uptake which is restored by exogneous IGF-I. Pyruvate kinase, neuron-specific enolase, malate dehydrogenase and creatine kinase are also significantly altered in IGF-I null brains, demonstrating that absence of IGF-I results in major disruption of cerebral metabolic pathways and supporting the hypothesis that endogenous IGF-I acts as the brain's insulin. Other studies in the IGF-I null mice have shown that the S-phase of the cell-cycle appears to be prolonged in the knockouts, providing novel insight as to the mechanism of IGF-I's growth promoting effects.

Studies on the role of the IGF system in reproduction have made substantial progress. Studies in the IGF-I knockout mouse have demonstrated that contrary to current views, IGF-I is not the mediator of estrogen's growth-promoting effects on the uterus, but rather is necessary for estrogen production. Other studies suggest that IGF-II deletion causes dwarfism primarily by attenuation of placental growth rather than by affecting the fetus directly.

PROJECT DESCRIPTION

Objectives/Methods:

The goal of this project is to understand the fundamental mechanisms regulating mammalian growth at the cellular and organismal level. To further this aim, this section focuses on the biological roles and mechanisms of action of insulin-like growth factors-I and -II (IGF-I and -II). Previous work from this lab has demonstrated the cell types expressing the different IGF system components and analyzed physiological changes in levels of gene expression in these different cell types and the nature of cellular functions associated with IGF expression. Original hypotheses derived from these studies are now tested in IGF system gene deletion mice and then compared and corroborated with observations in humans or non-human primates. The ultimate goal is to apply knowledge of IGF's biological roles in the diagnosis and treatment of disorders of human development and growth, including neoplasia.

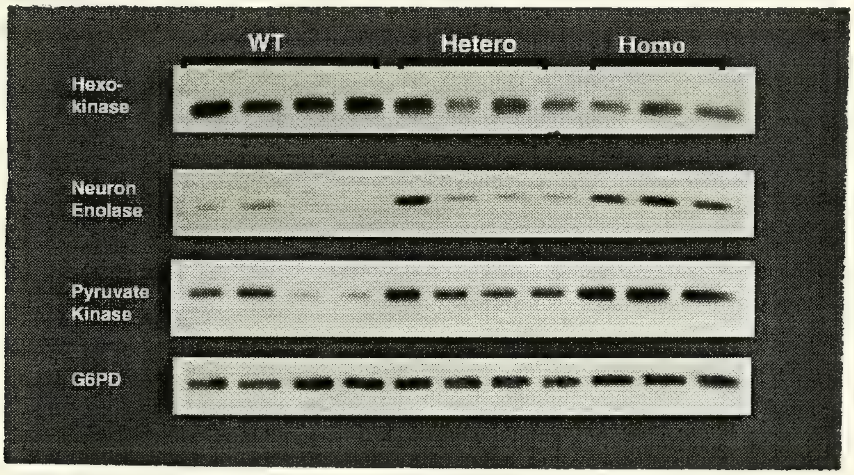
Major findings:

1) IGF action in the nervous system

The highly metabolically active brain relies almost exclusively on glucose to supply fuel. Despite the fact that the brain constitutes only about 2% of body weight, it accounts for over 20% of the body's glucose consumption. The manner in which brain glucose metabolism is regulated, however, is poorly understood. Little if any insulin is synthesized within the brain and very little crosses the blood brain barrier. Based our demonstration that neuroantomical patterns of insulin-like growth factor I (IGF-I) and IGF-I receptor expression track regional brain glucose utilization — it was hypothesized that locally produced IGF-I might serve to regulate neuronal glucose metabolism.

To evaluate this hypothesis, the expression of proteins involved in glucose transport and metabolism was compared in brains from targeted IGF-I gene deletion and wild type adult mice using western blotting. The neuron-specific glucose transporter, GT3, and the blood brain barrier glucose transporter, GT1, are increased in the IGF-I null brain. Hexokinase, the rate limiting enzyme in neuronal glucose utilization, was reduced to less than one third that found in wildtype brain and enzyme activity levels were also significantly reduced. In contrast, enzymes involved in the glycolytic generation of ATP (which are negatively regulated by ATP level) such as pyruvate kinase and neuron specific enolase, are up-regulated in IGF-I knockout brains by approximately 5- and 10-fold, respectively. Interestingly, another ATP-generating enzyme, CPK, was also increased in knock out brains. Values for heterozygote brains fell midway betweem wildtype and homozygous deletion brains in all these cases. Noninsulin-sensitive metabolic enzymes such as glucose-6-phosphate dehydrogenase (G6PD) and structural proteins such as MBP and GAP43 were not affected by IGF-I deletion.

Fig. 1 Metabolic enzymes in IGF-I null brains by immunoblotting.



In vivo studies of regional brain glucose utilization employing the 14C-2-deoxyglucose technique reveal a selective reduction in glucose uptake in the sensory projection centers (e.g., olfactory bulb and inferior colliculus) which express the highest IGF-I levels in normal adult brain. In vitro studies showed that glucose uptake is significantly decreased in synaptosomes from knockout brains and normalized by addition of IGF-I (Fig. 2).

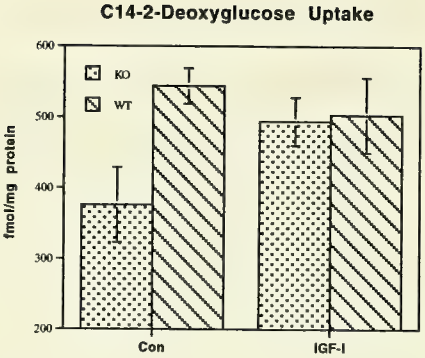


Fig. 2

These data support a fundamental role for local IGF-I production in regulation of brain glucose metabolism. By analogy with the known effect of insulin on glucose transporter function in the periphery, it is possible that GT3 shuttling from intracellular vesicle to neuronal plasma membrane surface is impaired in the absence of IGF-I. Alternatively, the defect in neuronal glucose uptake may be due primarily to the reduction in hexokinase expression in IGF-I knockout brains. The decrease in neuronal glucose transport and phosphorylation is predicted to result in diminished ATP production and compensatory up-regulation of ATP sensitive enzymes.

2. IGFs and the Reproductive system

a) Ovary

The group has previously reported that IGF-I mRNA is restricted to granulosa cells in a subset of preovulatory follicles in the rodent ovary and shown that IGF-I positive follicles are characterized by cell proliferation, FSH receptor (FSHR) expression and exclusion of c-fos and -jun expression. Recent work showed that FSHR mRNA is undetectable in the IGF-I null mouse ovary, suggesting that follicular IGF-I regulates granulosa cell FSHR gene expression and hence determines follicular growth and maturation in response to gonadotropin stimulation. This prediction has been confirmed in dynamic studies of the IGF-I knockout mouse, performed in collaboration with A. Efstradiatis' group at Columbia, showing that the IGF-I null female is gonadotropin resistant, remaining prepubertal despite a normal peripubertal rise in pituitary gonadotropins, and demonstrating resistance to ovulation induction by pharmacological treatment with gonadotropins.

This section's work has previously shown that human and rhesus monkey ovarys demonstrate a similar pattern of IGF system expression and that the primate pattern diverges very significantly from the rodent, such that studies in the mouse ßmay not be directly relevant to the human. Thus the group has pursued studies of the IGF system in rhesus ovaries. They have now shown that a specific IGF binding protein, IGFBP4, is selectively expressed in dominant follicles in the rhesus and also in the human ovary. The spatiotemporal patterns of IGFBP4 expression parallel LH receptor expression and granulosa cell luteinization, suggesting IGFBP4 expression is regulated by LH and that attenuation of IGF effect by this inhibitory binding protein regulates the progression of follicular development from the proliferative, E2 synthetic phase to the differentiated, P4 synthetic stage.

One of the fundamental differences between murine and primate ovary IGF system expression is that murine follicles selectively express IGF-I while primate follicles constitutively express IGF-II. The group hypothesized that the different patterns of IGF expression are related to the radically different ovarian cycles demonstrated by these species, with mice producing 10-15 ovulations every 4 days while primates produce one every month. To investigate this hypothesis, patterns of IGF system expression were examined in the porcine ovary, which represents a composite of the above patterns, with 10 or more ovulatory follicles produced every three weeks. Interestingly, both IGFs were detected in porcine follicles, with IGF-I displaying a selective and IGF-II a constitutive pattern of expression, suggesting that IGF-II expression is associated with the prolonged process of follicular development seen in larger species while IGF-I expression is associated with the development of multiple follicles each cycle.

b) Uterus

For many years it has been thought that uterine growth in response to E2 was due to the local production of IGF-I. This "estromedin hypothesis" was tested by treating IGF-I knockout and wildtype littermate controls with E2. Surprisingly it was found that uteri from both genotypes responded equally in terms of cell proliferation as determined by 3H-thymidine incorporation and growth in size over a 24 hour period. This finding appears to refute the view that IGF-I mediates E2's proliferation-inducing effects in the uterus.

c) IGF-II and placental growth

The IGF-II null mouse displays a proportional dwarfism which first becomes evident midway through gestation, at the time of placenta formation. Given IGF-II's pattern of genomic imprinting in which the gene is selectively expressed by the paternal allele, given the predominant role of paternal genes in placentation and our prior findings that IGF-II is expressed in paternally derived trophoblast tissues which give rise to the placenta, we hypothesized that this dwarfism may be due to a placental defect as opposed to embryo-autonomous somatic growth impairment. To test this view, mice impregnated by heterozygous deletant fathers (carrying half wild type and half paternal deletant dwarf embryos) were injected with 3H-thymidine on days 8, 9, 11, 12 or 14 of gestation and killed one hour later. Cell proliferation rate was significantly decreased and programmed cell death increased in placental labyrinths from the knockout mice, supporting the view that IGF-II regulates embryonic size by promoting placental growth.

d) Mammary gland

In a collaborative study with Dr. LeRoith, NIDDK, elderly female rhesus monkeys treated with GH by alzet pump for 7 weeks were noted to have mammary enlargement relative to saline treated animals. Histologic evaluation of the mammary tissue revealed a highly significant increase in epithelial cell proliferation rate as determined by detection of the Ki-67, proliferation specific antigen. This effect was particularly dramatic in follicular phase monkeys but was also present in luteal phase animals. The proliferative effect was positively correlated with circulating IGF-I levels; no relation to circulating estradiol or progesterone levels has been detected.

Proposed Course of Action:

1) IGFs and brain

The direct site of IGF-I action in regulation of neural bioenergetics will be investigated. An exofacial Glut3 photoaffinity label will be used in synaptosomal experiments to determine if IGF-I regulates translocation of endosomal GLUT3 to the plasma membrane in the same way that insulin regulates GLUT4. Additionally, the ultrastructural localization of GLUT3 will be compared in wildtype and knockout brains, to determine if this transporter is sequestered intracellularly in the absence of IGF-I. Alternatively, or possible in addition to regulating GLUT3, IGF-I may regulate brain hexokinase by inducing its synthesis and/or dephosphorylation (activation), as insulin does for another heoxokinase isoform in the periphery. Phosphorylation levels of brain hexokinase in IGF-I null brains will be compared to wildtype.

The changes in metabolic enzyme levels detected by immunoblotting will be further examined at the neuroanatomical level by enzyme histochemistry and at the genetic level by Northern and in situ hybridizaton analysis. As soon as we are able to generate a sufficient number of animals, the functional consequences of IGF-I deletion will be evaluated by behaviorial testing and by measuring responses to neurostressors such as ischemia, hypoglycemia and the aging process.

Further studies will compare IGF-I null versus wildtype littermate brains at the time of normal maximal IGF-I production (~postnatal day 9-10) for expression of putative mediators of IGF action, e.g., IRS-1. Differential display of mRNAs will be used to identify major targets of IGF-I regulation in brain which are not currently suspected.

2) IGFs and Reproduction
a) Ovary

The murine IGF-I knockout mouse model will be used to elucidate the mechanisms by which IGF promotes follicular maturation, estrogen biosynthesis and ovulation. Cell proliferation, survival and differentiation will be assessed using histological techniques and follicular culture will be employed to assess the effects of IGF-I repletion.

b) Uterus

The IGF-I knockout mouse uterus will be investigated further to determine what role if any local IGF-I production plays in mediating E2's effects on uterine growth or differentiation. If cell division rates are equal and growth starts out normally in response to E2, diminished cell survival may occur and result in growth failure over a period of days. Thus, E2 treatment will be sustained for 3 days and growth rates compared in wildtype and null mice, and uteri will be examined for evidence of increased apoptosis by in situ end-labeling, DNA laddering and expression of apoptosis-related proteins (Bax, bcl, Fas, ICE). It is possible that IGF-I mediates E2's stimulatory effect on uterine glycogen accumulation, so glycogen synthesis will be compared in baselin and E2-treated wildtype and IGF-I null mice uteri by histochemical techniques.

c) IGF-II and placenta

Cell proliferation and death rates will be assessed by cell type, i.e., cytotrophoblast vs syncitiotrophoblast. These same parameters will also be assessed in the embryo proper to determine whether the growth defect is, as hypothesized, confined to the placenta. The expression of cell cycle and programmed cell death regulators such as cyclin B, cdc2, p27kip1 bax and bcl will be evaluated by immuno- and northern blot analysis. Differential display will be used to probe for potentially novel genes mediating IGF-II action in placental development. The possibility that IGF-II expression regulates placental size by stimulating (maternal) vascular invasion will be tested by injection of gravid uterine horns in vivo with tetrazolium blue dye by the method of A. Psychoyos.

Significance:

The understanding of basic mechanisms regulating growth and reproduction is of obvious importance to the mission of NICHD. IGFs play a central role in these processes, and hence the elucidation of their effects in clinically relevant model systems is predicted to contribute significantly to the ability to diagnose and treat disorders of human growth and reproduction. There is no doubt, for example, that elucidation of the roles of IGFs in ovarian follicular development, placentation and brain metabolic function will have major therapeutic implications.

Publications:

Adesanya OO, Zhou J, Bondy CA. Sex steroid regulation of insulin-like growth factor system gene expression and proliferation in primate myometrium. J Clin Endocrinol Metab 1996;81:1967-74.

Adesanya OO, Zhou J, Bondy CA. Cellular localization and sex steroid regulation of insulin-like growth factor binding protein (IGFBP) mRNAs in the primate myometrium. J Clin Endocrinol Metab 1996;81:2495-2501.

Baker J, Hardy MP, Zhou J, Bondy CA, Lupu F, Bellve A, Efstratiadis A. Effects of an IGF-I null mutation on mouse reproduction. Mol Endocrinol 1996;10:903-18.

Bondy CA and Zhou J. Functional correlates of IGF system expression in the murine and primate ovary. In: LeRoith D, ed. The role of IGFs in ovarian physiology. Rome: Ares Serono Publications, 1996.

Reinhardt RR and Bondy CA. Differential cellular pattern of gene expression for two distinct cGMP-inhibited cyclic nucleotide phosphodiesterases in developing and mature rat brain. Neuroscience 1996;72:567-78.

Schoen TJ, Bondy CA, Zhou J, Dhawan R, Mazuruk K, Arnold DR, Rodriguez I, Waldbillig RJ, Beebe DC, Chader GJ. Differential expression and in situ localization of insulin-like growth factor binding protein-2 in developing chick ocular tissues. Invest Ophthalmol Vis Sci 1995;36:2652-62.

Zhou J, Adesanya OO, Vatzias G, Hammond JM, Bondy CA. Selective IGF system expression during porcine ovary follicular selection. Endocrinology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00631-07 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Neuroendocrine Control of the Stress Response

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	G. Aguilera	Head	SEP, DEB, NICHD
Others:	A. Kiss	Visiting Scientist	SEP, DEB, NICHD
	C. Rabadan-Diehl	IRTA Fellow	SEP, DEB, NICHD
	T. Ochedalski	Visiting Fellow	SEP, DEB, NICHD

COOPERATING UNITS *(if any)*

S. Lolait, LCB, NIMH, NIH

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Endocrine Physiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.1	3.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies of this laboratory demonstrated stress-induced expression of corticotropin-releasing hormone (CRH) receptors in the PVN, parallel to increases in CRH peptide expression. To test the hypothesis that CRH has a positive autoregulatory effect in the CRH neuron, experiments were performed in rats subjected to adjuvant-induced arthritis, chronic stress model associated with decreased CRH expression in the PVN. In these studies adjuvant injection failed to increase CRH receptors in the PVN, either after 7 days, the onset of the inflammation or at 14 days when severe inflammation is established. This finding supports the view that positive feedback by CRH is necessary for the enhanced CRH expression during chronic stress.

Studies on the regulation of CRH receptor in the pituitary showed that corticosterone injection at doses increasing plasma levels in the stress range, causes transient decreases in CRH receptor mRNA. This suggest that glucocorticoids contribute to the transient decrease in CRH receptor mRNA observed during the early stress response. While glucocorticoids regulate pituitary CRH receptors directly, previous studies have suggested that the transient decreases in CRH receptor mRNA following adrenalectomy are mediated by hypothalamic CRH release. This hypothesis was tested in rats subjected to PVN lesion or median eminence deafferentation to eliminate the source of hypothalamic CRH. While the lesions abolished CRH binding loss and the increases in POMC mRNA after long term adrenalectomy (4 days), only partially prevented the decrease in CRH receptor mRNA and increase in POMC expression after 18 hr adrenalectomy. The data show that increased hypothalamic CRH following glucocorticoid withdrawal is largely responsible for the changes in CRH receptors and POMC expression, and suggest the involvement of additional factors during the early phases after adrenalectomy.

Regulation of the HPA axis during chronic stress includes sensitization of the adrenal to ACTH. Studies in isolated adrenal fasciculata cells from repeatedly stressed rats revealed increased cAMP responses to ACTH indicating that increased activity of the ACTH receptor contributes to the enhanced responses. Increased steroidogenic capacity of the adrenal is mediated by increases in side chain cleavage (scc) enzyme activity and P-450scc mRNA levels, and hypertrophy of the zona fasciculata of the adrenal.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

The aim of this project is to characterize the neuroendocrine components of the adaptation to stress, with special emphasis in the mechanism of regulation of the hypothalamic pituitary-adrenal axis. This project involves characterization of receptors for the regulatory hormones, elucidation of the mechanism that regulate expression, synthesis and secretion of hypothalamic regulatory factors, elucidation of the molecular mechanisms that follow hormone receptor interaction, analysis of hormonal secretory patterns and CRH receptor regulation during development and manipulations of the hypothalamic-pituitary-adrenal axis. It is expected that this work will provide information on the physiological regulation of ACTH secretion and provide a basis for the understanding of pathological alterations of the hypothalamic-pituitary-adrenal axis.

Methods Employed:

The experiments are performed mainly in rats, and include both in vivo and in vitro procedures. In vivo experiments include application of various stress paradigms, prolonged infusions of CRH, VP and other regulators with osmotic minipumps, blood collection in conscious rats implanted with chronic intravascular catheters, implantation of icv cannulae and lesions of specific brain areas using stereotaxic procedures. In vitro procedures include measurement of CRH, VP and glucocorticoid receptors by conventional receptor assays and autoradiography, detection of neuropeptides by RIA and immunohistochemical techniques, preparation of enzyme-dispersed pituitary and brain cells, measurement of hormone release and intracellular messengers, measurement of adenylate cyclase and protein kinases, measurement of mRNA for several peptides, receptors and enzymes by northern blot, hybridization in solution and in situ, and molecular cloning techniques.

Progress Report::

Previous studies of this laboratory have demonstrated three types of responses of the hypothalamic-pituitary-adrenal axis during adaptation to chronic stress: a) desensitization of the ACTH to the primary stress, but hyperresponsiveness to a novel stimulus, b) sustained ACTH response to the primary stimulus and hyperresponsiveness to a novel stimulus, and c) minor ACTH responses to the primary stimulus and reduced sensitivity to a novel stress. Chronic stress paradigms associated with pituitary hyperresponsiveness showed activation of parvicellular CRH and CRH/VP neurons of the PVN, and increased VP/CRH secretion ratios. The activity of magnocellular neurons which provide VP to the neurohypophysis and peripheral circulation is unaffected by these stress paradigms.

In addition to the sensitivity of the hypothalamic pituitary responses, regulation of the adrenal sensitivity to ACTH and Ang II play and important role controlling circulating corticosteroid levels during chronic stress. Acute stress raises plasma levels of ACTH and Ang II, major regulators of adrenocortical steroidogenesis, resulting in increases of circulating corticosterone (in the rat, cortisol in humans) and aldosterone levels. However, a dissociation between plasma levels of steroids and their regulators is often observed during chronic stress, with unaltered plasma ACTH but increased A.M. corticosterone levels, and reduced plasma aldosterone levels with elevated PRA. The mechanism of this altered adrenal responsiveness was studied by analysis of ACTH and Ang II responses and the expression and activity of steroidogenic enzymes in the adrenal of rats subjected to repeated immobilization (2 hr/day for 14 days). Concomitant with reduced plasma aldosterone levels, maximum aldosterone responses to Ang II, ACTH nd 8-Br-cAMP were significantly decreased in isolated adrenal glomerulosa cells from repeatedly stressed rats. P450-aldosterone synthetase mRNA levels and binding of ^{125}I[Sar^{1},Ile^{8}]Ang II were significantly reduced in the adrenal zona glomerulosa of stressed rats. In contrast, the early biosynthetic pathway was enhanced in chronically stressed rats as indicated by increased pregnenolone production by isolated cells, and increased P450-side chain cleavage mRNA levels in the adrenal zona glomerulosa. Consistent with the elevated plasma corticosterone levels, pregnenolone and corticosterone responses to ACTH in isolated adrenal fasciculata cells from stressed rats were significantly increased. This was accompanied by increases in cAMP production suggesting an increase in ACTH receptors. While the zona fasciculata area was increased in stressed rats, levels of P450-side chain cleavage, but not those of 11-beta hydroxylase were significantly increased. Daily injection of ACTH (Cortrosyn s.c., 5, 4, 3, 2, 1 and 0.5 μg/day during days 1-2, 3-4, 5-6, 7-9, 10-12 and 13-14, respectively), to mimic the pattern of plasma ACTH during repeated immobilization, reproduced the changes in P450 mRNAs levels observed in adrenal from stressed rats. The half life of corticosterone in plasma measured with [^{3}H]corticosterone was significantly increased in stressed rats but not in ACTH injected rats. NADP-dependent 11-beta dehydrogenase activity in liver homogenates was also decreased in stressed rats suggesting that decreased glucocorticoid inactivation and clearance rates contribute to the increased corticosterone levels during chronic stress. This study shows that chronic stress leads to a) inhibition of mineralocorticoid secretion due to inhibition of the late biosynthetic pathway, and b) increased circulating glucocorticoids due to increased ACTH receptor activity, expression and activity of the early pathway, and decreased glucocorticoid inactivation and clearance. The data suggest that altered adrenal glomerulosa and fasciculata function, but not changes in 11-beta dehydrogenase activity, are the result of repeated adrenal exposure to high ACTH levels during chronic stress.

Work of this laboratory has shown a stress-specific induction of hypothalamic CRH receptors, with increases in the parvicellular PVN during physical-psychological stress and in the magnocellular PVN and in the SON after osmotic stimulation. The physiological role of CRH in the PVN is still unclear but the association of increases in both CRH receptor and CRH peptide expression suggests that CRH has a positive autoregulatory role in the CRH neuron. To test this hypothesis, research during the current year was extended to study CRH receptor mRNA levels in the PVN during experimental inflammatory stress, condition in which the expression of CRH peptide in the PVN is decreased.

The expression of CRH in the PVN and CRH receptor mRNA in the PVN and anterior pituitary was studied during development of adjuvant-induced arthritis in Piebald-Viral-Glaxo rats, using in situ hybridization techniques. As previously shown, 14 days after adjuvant injection, basal and immobilization stress-stimulated CRH mRNA levels in the PVN were significantly lower than in controls. However, 7 days after injection, preceding the onset of inflammation, the increase of CRH mRNA following immobilization was significantly higher than in control rats. In contrast to other chronic stress paradigms, inflammation stress failed to induce CRH receptor mRNA in the PVN, either at 7 days, or at 14 days after adjuvant injection, when severe inflammation is present. The ability of acute immobilization to induce CRH receptor mRNA in the PVN was not affected 14-days after adjuvant injection but parallel to the CRH peptide mRNA response it was markedly potentiated at 7 days. POMC mRNA levels in the anterior pituitary increased significantly only 14 days after adjuvant injection, and they were unaffected by 1 hr immobilization. While CRH binding in the pituitary decreased significantly 14 days after adjuvant injection, CRH receptor mRNA was unchanged, suggesting that the decrease in binding is due to receptor occupancy and internalization rather than to a decrease in receptor synthesis. The study shows biphasic hypothalamic responses to acute stress during development of adjuvant-induced arthritis, with enhanced CRH peptide and CRH receptor mRNAs responses at 7 day, preceding the onset of inflammation, and blunted CRH mRNA responses at 14 days at the height of the inflammatory response. The lack of CRH receptor expression in the PVN in this model of chronic inflammation stress supports the view that positive feedback regulation by CRH is necessary to maintain enhanced CRH expression during chronic stress.

Corticotropin releasing hormone (CRH) regulates ACTH secretion and POMC transcription by interaction with type-1 CRH receptors in the pituitary corticotroph. Studies during the past year showed complex regulatory effects of glucocorticoids on pituitary CRH receptor mRNA. While dexamethasone administration causes sustained decreases in POMC mRNA and CRH binding, it only transiently decreases CRH receptor mRNA, suggesting that glucocorticoids regulate CRH receptor expression at the transcriptional and post-transcriptional levels. Extension of these studies showed similar regulatory effects on CRH receptor mRNA after administration of the natural glucocorticoid corticosterone, at doses increasing plasma levels in the stress range. This indicates that increases in circulating glucocorticoids play a role regulating pituitary CRH receptors during stress.

Northern blot and in situ hybridization studies in this laboratory have shown transient decreases in pituitary CRH receptor mRNA after adrenalectomy, falling to about 50 of the control levels after 18 hr, and returning to control values after 6 days. While glucocorticoids have direct regulatory actions of pituitary CRH receptor mRNA, it is likely that the inhibition after short term adrenalectomy is indirect, mediated by glucocorticoid dependent changes of hypothalamic regulators. To test this hypothesis studies were performed in rats subjected to adrenalectomy after suppression of hypothalamic CRH and VP by PVN lesions or median eminence deafferentation by hypothalamic anterolateral cuts (ALC). PVN lesions or ALC, resulting in undetectable irCRH and VP in the external zone of the median eminence, had no effect on basal pituitary POMC hn RNA or mRNA, CRH binding and CRH receptor mRNA levels. Adrenalectomy for 18 hr or 4 days increased POMC hnRNA and mRNA, and decreased CRH binding. CRH receptor mRNA levels decreased by about 50% 18 hr after adrenalectomy but returned to basal by 4 days. While the fall in CRH binding after 18 hr or 4 days adrenalectomy was fully prevented by PVN lesions or ALC, the decrease in CRH receptor mRNA levels after 18 hr adrenalectomy was only partially prevented (80% and 72% the control values). Similarly, ALC prevented adrenalectomy-induced elevation of POMC hnRNA and mRNA after 4 days, but not the early increase after 18 hr. The data indicate that while increased release of hypothalamic regulators induced by glucocorticoid withdrawal (mainly CRH and VP) leads to CRH receptor binding loss following adrenalectomy, it only partially explains the early decrease in CRH receptor mRNA. The inability of CRH antibody to prevent the transient decrease in CRH receptor mRNA and the initial increase in POMC transcription, renders it unlikely that small amounts of residual CRH in the median eminence are responsible for this effects, and suggest the involvement of other hypothalamic regulators.

Significance to Biomedical Research and the Program of the Institute:

Exposure to different degree of stressful stimuli is a prevalent problem in society. Stressful stimuli are integrated in the central nervous system and lead to a number of responses that include behavioral, visceral and hormonal adaptation. The changes in the hypothalamic-pituitary axis during prolonged stress can result in alteration of the secretion of most pituitary hormones with consequent alterations in metabolic, immune and reproductive function. Elucidation of the physiological mechanisms involved in the stress response will be important for the prevention and management of clinical conditions that involve disorders of neuroendocrine regulation.

Proposed Course:

Studies on the regulation of pituitary CRH and VP receptor expression will be extended to the molecular mechanisms. Initial studies will determine the second messengers involved in the regulation of the mRNAs of these receptors in primary cultures of pituitary cells and in the cell line ATt-20. The cloning of the promoter region of the CRH receptor and VP receptor genes to study the transcriptional regulation of these receptors is a priority. A single genomic clone containing about 10kb upstream the 5'untranslated region of the VP receptor has been already obtained and is in the process of being sequenced. Studies are also in progress to determine the mechanisms involved in the dramatic changes in CRH receptor expression in the PVN during stress. The physiological role of CRH in the regulation of the PVN function during stress is under study using CRH antagonists and antisense oligonucleotides to block the expression of the receptor.

Studies on the role of glucocorticoid feedback will be extended to the differential analysis of changes in glucocorticoid and mineralocorticoid receptors in pituitary and brain, as well as changes in 11ß-dehydrogenase, enzyme which determines the interaction of glucocorticoids with the receptors. The expression of transcription factors which regulate the interaction of glucocorticoid receptors with glucocorticoid regulatory elements in the POMC gene will be studies in pituitary cells isolated from control and stressed rats and in cell lines.

Publications:

Aguilera G, Kiss A, Lu A, Camacho C. Regulation of adrenal steroidogenesis during chronic stress. Endocrine J, in press.

Aguilera G, Rabadan-Diehl C, Luo X and Kiss A. Regulation of pituitary ACTH secretion during stress: role of corticotropin releasing hormone and vasopressin receptors. In: Stress: molecular genetic and neurobiological advances. Gordon and Breach, New York, in press.

Jezova D, Kiss A, Aguilera G. Studies on the mechanisms of the atypical plasma vasopressin responses to stress in chronically adrenal demedullated rats. Stress, in press.

Johnson MC, Devotto LRetamales I, Kohen P, Troncoso JL, Aguilera G. IGF-I and IGF Ireceptor expression and stimulation of estradiol secretion in human corpus luteum. Fertility and Sterility 1996;65:489-94.

Kiss A, Palkovits M, Aguilera G. Extrahypothalamic regulation of corticotropin releasing hormone (CRH) and CRH receptor mRNA in the hypothalamic paraventricular nucleus in the rat. J Neuroendocrinol 1996;8:103-12.

Luo X, Kiss A, Rabadan-Diehl C, Aguilera G. Regulation of hypothalamic and pituitary corticotropin releasing hormone receptor mRNA by adrenalectomy and glucocorticoids. Endocrinology 1995;136: 3877-83.

Makara GB, Kiss A, Lolait SJ, Aguilera G. Hypothalamic pituitary corticotroph function following shunting of magnocellular vasopressin to the hypophyseal portal circulation. Endocrinology 1996;137:580-6.

Rabadan-Diehl C, Kiss A, Camacho C, Aguilera G. Regulation of messenger ribonucleic acid for corticotropin releasing hormone receptors in the pituitary during stress. Endocrinology, in press.

Rabadan-Diehl C, Lolait SJ, Aguilera G. Regulation of vasopressin V1b receptor mRNA during stress in the rat. J Neuroendocrinol 1995;7:9903-10.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00632-07 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Physiological Actions of the Renin-Angiotensin-System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Aguilera	Head	SEP, DEB, NICHD
Others:	A. Kiss	Visiting Scientist	SEP, DEB, NICHD

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section Endocrine Physiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to study physiological and pathological aspects of the renin-angiotensin system, with emphasis on its role on circulatory homeostasis and development. During the past year studies have focused on a) the role of type-1 angiotensin II (Ang II) receptors located in parvicellular CRH and CRH/VP cells in the hypothalamic paraventricular nucleus (PVN), b) the control of mineralocorticoid secretion during development.

a) Previous studies of this group showed that AT1 receptors in the PVN are located in neurons expressing corticotropin releasing hormone (CRH) in the parvicellular subdivision. To determine the physiological role of these receptors, the effects of acute stress on the hypothalamic pituitary adrenal (HPA) axis and sympathetic activity were studied in rats subjected to icv injection of Losartan. Central AT1 receptor blockade had no effect on stress-induced elevations in plasma ACTH and corticosterone, CRH mRNA and CRH receptor mRNA in the PVN. However, the increases in plasma epinephrine and norepinephrine in response to stress were significantly smaller in Losartan injected rats.

b) In the adult, mineralocorticoid secretion in the adrenal zona glomerulosa is characterized by: 1) high dependance on Ang II, 2) biphasic effects of ACTH, with stimulation after initial exposure followed by inhibition, and 3) minor inhibition by glucocorticoids. Studies during development showed that while aldosterone responses in 7-day old rats are markedly insensitive to Ang II in vivo, they are similar to those in adults in vitro. Aldosterone responses to ACTH are normal in vivo and in vitro. This indicates that the steroidogenic capacity of the adrenal is normal in immature rats, but an endogenous factor inhibits the Ang II action. A novel finding was that glucocorticoids markedly inhibit aldosterone secretion in vivo but not in vitro. This effect is mediated by inhibition of aldosterone synthetase expression, and it was not prevented by ACTH injection. These studies show marked developmental changes in the mechanism of regulation of aldosterone secretion.

Project Description:

Objectives:

The purpose of this project is to study the role of the renin-angiotensin-system in mammalian physiology with special emphasis on its role in the homeostasis and growth and development. Studies include characterization and mapping of the distribution of Ang II receptors subtypes and their function in the nervous system, endocrine organs and other peripheral tissues, and elucidation of the molecular mechanisms involved in the regulation of cellular function by the peptide. Studies on the control of mineralocorticoid secretion include analysis of the interaction of Ang II with other regulators, at the systemic and cellular level and to define the mechanisms responsible for changes in adrenal sensitivity to the peptide during changes in sodium intake.

Methods Employed:

The majority of experiments are performed in the rat, and involve both in vivo and in vitro procedures. In vivo procedures include modifications of dietary electrolytes, administration of Ang II and inhibitors of the renin-angiotensin system and blood sampling in conscious rats with chronically implanted intravascular catheters. In vitro procedures include preparation of enzyme-dispersed fetal fibroblasts and adrenal glomerulosa cells, measurement of Ang II receptors in dispersed cells, membrane rich fractions and slide mounted tissue sections by conventional assays and by autoradiography; measurement of steroid production and intracellular messengers in isolated cells under basal and stimulated conditions; analysis of cellular proteins by gel electrophoresis and immunoblot techniques, and determination of mRNA by Northern blot, hybridization in solution and in situ hybridization.

Major Findings:

Angiotensin II is a major regulator of adrenal mineralocorticoid secretion and blood pressure. In addition, components of the renin angiotensin are present in the brain an a number of peripheral tissues where acts as a neurotransmitter or as a paracrine regulator. The effects of Ang II are mediated by plasma membrane receptors located in the adrenal zona glomerulosa and other target tissues. Two major subtypes of Ang II receptors have been identified, AT1, which are coupled to calcium-phospholipid dependent signalling systems, and AT2, with yet undefined actions. Previous research of this laboratory have described the topographic localization and characterization of Ang II receptors subtypes in the brain and peripheral tissues. These studies have shown that the major Ang II receptor subtype in the hypothalamus and in the adrenal zona glomerulosa is AT1 receptor.

Studies of this group using double staining in situ hybridization techniques have shown that AT1 receptors in the hypothalamic paraventricular nucleus (PVN) are located primarily in corticotropin releasing hormone (CRH) neurons of the parvicellular subdivision. AT1 receptor expression in the PVN undergo marked upregulation following stress and glucocorticoid administration suggesting a role for Ang II in the central stress response. Further studies were performed during the last year to determine the physiological role of Ang in PVN function, by analysis of the effects of stress on CRH mRNA and CRH receptor mRNA in the PVN, and plasma levels of ACTH and catecholamines following central AT1 receptor blockade by icv injection of 25 μg of the specific antagonist Losartan. AT1 receptor blockade had no effect on stress induced CRH mRNA or CRH receptor mRNA in the PVN, or in stress stimulated ACTH secretion. However, stress caused significantly smaller elevations in plasma epinephrine and norepinephrine levels in Losartan treated rats. The data indicates that central AT1 receptors play an important role in the sympathetic responses, but not in the regulation of the hypothalamic pituitary adrenal axis to acute stress.

Studies on the role of Ang II during development were focused on the regulation of mineralocorticoid secretion in the immature rat (7-day). Studies in vivo showed that in contrast to adult rats, aldosterone responses to Ang II were markedly insensitive in 7-day old rats; levels were unchanged after 5 μg/kg, and increased 2 to 12-fold after 50 μg/kg Ang II. On the other hand, plasma aldosterone responses to ACTH were normal, and as in the adult, prolonged ACTH administration (4 days) reduced basal and Ang II-stimulated aldosterone secretion. In contrast, aldosterone responses to Ang II in collagenase-dispersed adrenal glomerulosa cells from 7 day old rats were similar to those in adult rats suggesting that a circulating or paracrine inhibitory factor reduces the sensitivity of the adrenal glomerulosa to Ang II in the immature rat.

A novel and unexpected finding was a marked inhibitory effect of glucocorticoids on adrenal glomerulosa function in 7-day old rats. In vivo, injection of 200 µg/kg dexamethasone, reduced basal serum aldosterone below the assay detection limit, and attenuated the responses to 50 ug/kg Ang II by 1 to 4 h, and abolish them by 18 h. In-vitro preincubation with dexamethasone for 2 hr did not decrease basal or stimulated aldosterone production by dispersed 7d glomerulosa cells, suggesting that the inhibitory effect in vivo is indirect. In situ hybridization analysis of mRNA levels of steroidogenic enzymes in the adrenal zona glomerulosa revealed a time dependent decrease in P-450 aldosterone synthetase mRNA (91±3%, 77±6%, 60±13% and 38±19% of control at 1,4,8, and 16h, respectively), and no significant changes in P-450 side chain cleavage and P-45011β mRNA levels, 18 hr after dexamethasone.

The decrease in aldosterone synthetase mRNA levels after dexamethasone injection was not due to inhibition of ACTH secretion because it was not prevented by a physiologic replacement dose of ACTH.
The data shows marked developmental changes in the responsiveness of the adrenal glomerulosa to regulatory hormones and glucocorticoids.

Significance to Biomedical Research and the Program of the Institute:

The prominence of Ang II receptor expression during crucial periods of fetal and postnatal growth open new perspectives about the importance of the renin-angiotensin system during development. Elucidation of the function of these novel receptors during development is of critical importance, especially in view of the increasing clinical use of drugs that affect the function of the renin-angiotensin system. The studies on the central actions of Ang II are likely to uncover novel regulatory mechanisms in neuroendocrine function, including modulatory actions of the activity of the HPA axis, growth and reproductive function. In addition elucidation of the mechanism of regulation of adrenal glomerulosa cell function is relevant to understanding the pathogenesis and treatment of disorders involving alterations of mineralocorticoid secretion. Understanding the mechanisms of neonatal adrenal function has considerable importance in human physiology. Glucocorticoids such as dexamethasone are commonly used in the medical management of the mother and fetus, and also in the neonatal nursery. In addition, this knowledge may help to resolve unanswered questions regarding adrenal responsiveness to peptides such as Ang II in the adult.

Proposed Course:

Future emphasis in this project will be placed in the elucidation of the neuroendocrine functions of Ang II. Based on the topographic localization of Ang II receptor mRNA in the PVN, the hypothesis to be tested is that Ang II has a modulatory effect on the function of the CRH neuron, and possibly on somatostatin and dopaminergic neurons of the PVN. Further in situ hybridization histochemistry studies will be performed to determine whether Ang II receptors are expressed in somatostatin and dopaminergic neurons in the PVN. The function of Ang II in the regulation of the HPA axis, growth hormone and prolactin secretion will be studied using Ang II receptor blockade with specific Ang II antagonists and antisense oligonucleotides during neuroendocrine manipulations, and by analysis of changes in Ang II receptor expression in relevant brain areas during manipulations of the HPA axis or gonadal hormones in male and female rats.

Publications:

Aguilera G and Kiss A. Regulation of the hypothalamic pituitary adrenal axis and vasopressin secretion: role of angiotensis II. In: Recent advances in cellular and molecular aspects of angiotensin receptors. Raizada MK, Phillips MI, Sumners C, eds. Plenum Press, New York, 1996;105-12.

Aguilera G, Kiss A, Luo X. Increased expression of type-1 angiotensin II receptors in the hypothalamic paraventricular nucleus following stress and glucocorticoid administration. J Neuroendocrinol 1995;7:775-83.

Aguilera G, Kiss A, Luo X and Sunar-Akbasak B. The renin-angiotensin system and the stress response. Ann NY Acad Sci 1995;771:173-86.

Aguilera G, Kiss A and Sunar-Akbasak B. Hyperrreninemic hypoaldosteronism following chronic stress in the rat. J Clin Invest 1995;96:1512-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD-00633-06 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ovarian folliculogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	L.M. Nelson	Senior Clinical Investigator	SWH, DEB, NICHD
Others:	Z.B. Tong	Visiting Fellow	SWH, DEB, NICHD
	J. Anasti	Clinical Associate	SWH, DEB, NICHD
	R. Maity	Visiting Fellow	SWH, DEB, NICHD

COOPERATING UNITS *(if any)*

J. Dean, Mammalian Developmental Biology Section, Laboratory of Cellular and Developmental Biology, NIDDK; R. Caspi, Laboratory of Immunology NEI

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Sectionon Women's Health

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project seeks to improve the clinical care available to patients with disorders of ovarian follicle function and ovulation through research using animal models and clinical protocols. In pursuing this goal, we expect to expand understanding of the ovarian follicle in health and disease. We have focused on premature ovarian failure, a condition that prematurely terminates ovarian function and fertility in 1% of women. We have found that femoral neck bone density is significantly reduced in women with premature ovarian failure. Surprisingly, more than one-half of our patients had a bone density low enough to cause a threefold increase their risk of fracture. Inappropriate luteinization of graafian follicles appears to be a major pathophysiologic mechanism in patients with karyotypically normal spontaneous premature ovarian failure. Strikingly, we found luteinized graafian follicles in all the antral follicles we biopsied in 6 patients with karyotypically normal spontaneous premature ovarian failure. Therefore, luteinized graafian follicles account for at least 60% of the antral structures imaged (95% confidence limit). We have particular interest in autoimmune ovarian failure. Using an animal model, we found that autoantibodies from mice with experimental autoimmune oophoritis bind to a 120 kd protein that is specific to the oocyte cytoplasm. By screening a mouse ovarian cDNA expression library with this serum, we identified a novel oocyte-specific gene that may represent the inciting antigen in this disease. By using transgenic technology we will investigate the function of this novel gene, and determine if the novel oocyte-specific-expression gene produces the inciting ovarian antigen in murine experimental autoimmune oophoritis. Future clinical directions involve developing strategies to increase bone density in women with premature ovarian failure and a treatment to prevent inappropriate follicle luteinization in patients with premature ovarian failure. We will also expand our efforts to include a clinical study of polycystic ovary syndrome. We have initiated a treatment protocol to test the hypothesis that the hyperandrogenism associated with polycystic ovary syndrome impairs follicular development by a receptor-mediated mechanism.

Project Description:

Objectives:

This project seeks to improve the clinical care available to patients with disorders of ovarian follicle function. We approach this goal from a broad perspective, aiming to advance knowledge of ovarian follicle physiology and pathophysiology through research using animal models of human reproductive disorders, and through clinical protocols evaluating diagnostic and therapeutic methods for these patients. The consolidating goal here is to develop methods to restore ovulation in patients currently unresponsive to available therapies. In the process of pursuing these goals, we expect to expand essential understanding of the ovarian follicle in health and disease. We now have specific interest in patients with premature ovarian failure. Approximately one percent of women experience ovarian failure prior to age 40. Although symptoms related to estrogen deficiency in these patients can be treated with estrogen replacement therapy, there is no known treatment to restore ovulatory function and make these women fertile. This problem has had greater impact in recent years. As modern women more commonly delay childbearing, more women will experience infertility as the most significant problem related to premature ovarian failure.

Our specific research objectives during the current period were directed along three main avenues.

1. Clinical research in premature ovarian failure
   a) Examine bone density in a group of these patients.
   b) Evaluate treatments for patients with autoimmune oophoritis.

2. Basic research to develop clinical diagnostic markers for autoimmune premature ovarian failure
   a) Explore antibodies against the zona pellucida as a marker for autoimmune premature ovarian failure.
   b) Examine the frequency of antibodies against human recombinant thyroid peroxidase and gastric parietal cells in patients with premature ovarian failure.

3. Basic research in murine post-thymectomy autoimmunity, an animal model of autoimmune premature ovarian failure
   a) Characterize the inciting ovarian antigen in this model.
   b) Define how neonatal thymectomy perturbs the developing immune system so as to induce autoimmunity.
   c) Evaluate natural killer (NK) cell activity and the mouse major histocompatibility system (H2) as determining factors in the strain-specific susceptibility to post-thymectomy autoimmune oophoritis.

Methods Employed:

Clinical premature ovarian failure

Premature ovarian failure is a condition causing amenorrhea, hypoestrogenism, and elevated gonadotropins in women less than age 40. Patients with known abnormal karyotypes or evidence to suggest iatrogenic ovarian failure are not included in our studies.

Patients with premature ovarian failure are evaluated clinically to include: measurement of sex steroid hormones, gonadotropins, free T4, and thyroid stimulating hormone by radioimmunologic methods; karyotype analysis on cultured lymphocytes; Human Leucocyte Antigen (HLA) analysis by immunophenotyping using standard microcytotoxic typing with locally prepared HLA-DR1-10 antiserum trays; determination of bone density by dual energy x-ray absorptiometry; and evaluation of ovarian structure by ultrasonography. Patients may also undergo a three hour glucose tolerance test and ACTH stimulation test. Autoimmune activity is determined by immunohistochemistry, hemagglutination assay, and enzyme linked immunosorbent assay. Antiovarian antibodies in women are detected by indirect immunofluorescence using M. cynomolgus ovary as tissue substrate. These clinical investigations involve the participation of either healthy human subjects or patients.

Previous investigators have used ovarian wedge biopsy in the evaluation of premature ovarian failure. However, wedge biopsy was originally developed as a technique used in polycystic ovarian syndrome, a condition associated with abnormally large ovaries. Patients with premature ovarian failure have small ovaries, and wedge biopsy in this condition has tremendous sampling error. Therefore, rather than performing wedge biopsy at a random time, we have performed directed ovarian follicle biopsy when an ovarian antral follicle was visualized by pelvic ultrasound. Ovarian biopsy is performed under general anesthesia by minilaparotomy or laparoscopy. Biopsy specimens are processed and stained routinely with hematoxylin and eosin, and immunohistochemical studies are performed to detect lymphocytic infiltration.

Murine post-thymectomy polyglandular autoimmunity

Thymectomy performed two to four days after birth induces murine polyglandular autoimmunity in a strain-specific manner. Mice may develop autoimmune thyroiditis, autoimmune gastritis, or autoimmune oophoritis, a constellation of autoimmunity that is seen in the clinic. We work primarily with (C57/BL6 x A/J)F1 hybrids (B6A) because 90% of B6A mice develop experimental autoimmune oophoritis after neonatal thymectomy.

To characterize the autoantibodies in murine experimental oophoritis we use immunoblotting. Ovarian proteins are prepared for electrophoresis on SDS-PAGE gel, transferred to nitrocellulose membrane, incubated with sera from thymectomized or control mice, and developed with secondary antibody using immunoperoxidase. To identify the ovarian proteins that bind antibody, we screen a mouse ovarian cDNA library with serum from mice with high-titer ovarian autoantibodies.

Previous cell transfer studies, using cells from spleens of normal adult mice, have shown that mice thymectomized as neonates are deficient in a CD4+ T cell population that can prevent autoimmunity. We seek to determine if this is a deficiency in CD4+ T helper 1 (TH1) or T helper 2 (TH2) activity. To characterize CD4+ TH1 and TH2 activity, we stimulate CD4+ splenic lymphocytes in vitro by engaging CD3 and CD28 with specific antibodies. We measure interferon-gamma (INF-gamma) production, a measure of CD4+ TH1 cell activity, by enzyme-linked immunosorbent assay (ELISA). We measure interleukin-4 (IL-4) production, a measure of TH2 cell activity, by utilizing a proliferation assay employing an IL-4-dependent cell line. We enrich for CD4+ T cells by magnetic separation and confirm the separation by flow cytometry. To characterize the location of the inciting ovarian antigen in this model we stain ovaries with immunoperoxidase immunohistochemistry using monoclonal antibodies to CD45, a cell surface protein specific to mouse leukocytes. We evaluate the importance of the mouse major histocompatibility system by performing neonatal thymectomy on congenic strains of mice that differ only at the H2 locus.

Major Findings:

1. Clinical premature ovarian failure

    a. Bone density

We found that, based on bone density measurements, over one-half of our patients with premature ovarian failure have a threefold increased risk of hip fracture. This is despite the fact that 85% of these patients had been taking estrogen replacement therapy. We found that 48 of 89 women with premature ovarian failure (54%) had a bone density at the femoral neck less than one standard deviation below the mean of normal age-matched women ($p<0.001$, chi square goodness of fit test). A bone density less than one standard deviation below the mean is associated with a threefold increase in the risk of fracture.

b. Treatment of autoimmune premature ovarian failure

Two previous studies, one by our laboratory and one by a laboratory in Boston, have demonstrated that patients with premature ovarian failure as a group have increased T lymphocyte activation in peripheral blood. This suggests autoimmunity plays a role in a substantial portion of these patients. However, we have no serum marker to accurately identify individual women who have premature ovarian failure on an autoimmune basis.

As part of a controlled study evaluating alternate-day prednisone therapy for patients with biopsy-proven autoimmune oophoritis, we biopsied developing antral ovarian follicles in six women. Despite our use of sensitive immunohistochemical methods, we did not find autoimmune oophoritis in any ovarian follicle biopsies performed on these patients. We have, therefore, modified our patient selection criteria to include patients with other evidence to suggest autoimmunity. Also, based on our findings in the mouse model, we suspect the disease can sometimes be sequestered in the ovarian hilum. Therefore, we have modified our ovarian biopsy technique. We are currently evaluating a novel laparoscopic ovarian biopsy strategy that will be certain to obtain a portion of the ovarian hilum.

2. Diagnostic markers for autoimmune ovarian failure

a. Zona pellucida antibodies

Strikingly, we found that approximately one-third of normal women (n=26) had these antibodies. Thus, the test is not helpful to women with premature ovarian failure. However, because one-half of our patients with premature ovarian failure had these antibodies, we attempted to improve the specificity of the test. We could not improve the test by using higher serum dilutions or simplified outcome measures.

We have also employed human recombinant ZP3 to create an ELISA system for detecting anti-ZP3 antibodies. We tested serum that contained antibodies against the zona pellucida as determined by immunofluorescence in this system but detected no antibody with the recombinant protein. Therefore, it appears likely that the antibodies are directed against either ZP-1 or ZP-2, but not ZP-3.

We are encouraged, however, by that fact that we did not detect zona pellucida antibodies in any of 26 men ($p< 0.001$). This is intriguing. This finding supports recent proposals that pre-B cells undergo positive selection directed by the presence of surface heavy chain with low affinity to autoantigen. It appears then, that men, lacking this self antigen, fail to provide positive selection for these pre-B cells clones. Also, this finding suggests that our detection system is specific for zona pellucida, and that further refinement of the assay system by using other pure human recombinant zona pellucida protein might be useful.

b. Thyroid peroxidase and gastric parietal cell antibodies

We found that women with karyotypically normal spontaneous premature ovarian failure have a significantly higher incidence of antibodies against human thyroid peroxidase and gastric parietal cells as compared to age-matched normal women. The constellation of autoimmunity seen in murine post-thymectomy autoimmune oophoritis includes autoimmunity against the thyroid and gastric parietal cells. Therefore, we determined whether a similar constellation of autoimmunity was present in patients with premature ovarian failure. Our finding suggests this mouse model may indeed have expression in human pathology, and studies to define the inciting antigen in this mouse model may have important implications to women with premature ovarian failure.

Murine post-thymectomy polyglandular autoimmunity

a. Inciting ovarian antigen

We have identified a novel oocyte-specific gene. Previously, we found that autoantibodies from mice with autoimmune oophoritis bind to a 120 kd protein that is specific to the oocyte cytoplasm. We identified the novel gene by screening a mouse ovarian cDNA expression library with this serum. We have demonstrated that the message is specific to the ovary by RNAse protection assay, and we have demonstrated that the message is specific to the oocyte by in situ hybridization. These mice not only develop high-titer autoantibodies specific to oocytes, but ultimately the ovaries become fibrotic and devoid of primordial follicles. These findings implicate the oocyte as a primary target of the autoimmune process.

We have shown that the inciting antigen in murine experimental autoimmune oophoritis appears to be produced independently of gonadotropin stimulation of the ovary. These findings are consistent with the hypothesis that the inciting antigen is present in oocytes of primary follicles, as suggested by the oocyte specificity of the high-titer serum. Primary follicle development does not require gonadotropin stimulation. By administering gonadotropin stimulation to neonatally thymectomized mice we did not exacerbate existing disease, or induce an earlier onset of severe disease. Furthermore, by administering gonadotropin suppression to neonatally thymectomized mice we did not reduce the degree of lymphocytic infiltration or oocyte destruction.

b. Perturbing the neonatal immune system

Adoptive transfer of CD4+ splenic lymphocytes from normal adult mice prevents the disease from developing in neonatally thymectomized mice. The mechanisms of this regulation, and the reason why the timing of neonatal thymectomy is so critical, are unclear.

We have findings to suggest that 1) neonatal thymectomy induces autoimmunity by impairing development of T helper type 1 (Th1) regulation, and 2) the postnatal shift to normal adult Th1/Th2 balance is established by a thymus-dependent process. These results hold implications for the pathogenesis, and possibly for the therapy of autoimmune polyglandular failure in humans.

Strikingly, we found that splenic CD4+ cells from adult mice thymectomized as neonates produced an inappropriate neonatal-like Th2 predominant response (high levels of IL-4 and low levels of interferon gamma). Cells from adult mice sham-operated as neonates produced the expected Th1 predominant response seen normally in adult mice (low levels of IL-4 and high levels of interferon gamma).

We also found that administration of interleukin-12, a key cytokine that promotes CD4+ T cell differentiation towards the Th1 phenotype, restored the adult-like Th1 pattern of mice thymectomized as neonates, and ameliorated the development of autoimmune oophoritis. We found that postnatal adoptive transfer of adult splenocytes to mice thymectomized as neonates had the same effect.

c. NK cell activity and the major histocompatibility complex

During the current period we showed that mice thymectomized as neonates have reduced natural killer (NK) cell activity. IL-12 also stimulates NK cells, and increases their production of INF-gamma. This is an additional indirect mechanism by which IL-12 promotes CD4+ cell differentiation toward Th1. IL-12, administered after neonatal thymectomy, appears to in some way compensate for the lack of thymic function. This raises the possibility that the thymus effects the change in the periphery by secretion of IL-12. The message for both the p35 and p40 chains of IL-12 are produced in mouse thymus.

By using congenic strains of mice in a well-controlled experiment, we also showed that genetic background plays a more important role than H-2 haplotype in the development of post-thymectomy autoimmune oophoritis. The availability of congenic strains of mice makes it possible to separate the effects of the genetic background and the specific H-2 major histocompatibility complex. "A" mice (A/J) are highly susceptible to post thymectomy autoimmunity, whereas "B" mice (C57 BL10) are relatively resistant. We showed that A.By mice developed severe autoimmune oophoritis equivalent to A/J mice. A.By mice have the "A" genetic background, but the "b" H-2 major histocompatibility complex. Therefore, severe disease can develop in "A" mice despite the absence of the "a" H-2. Furthermore, we found that B10.A mice, which have the "B" background and the "a" H-2, did not develop disease. Therefore, the presence of the "a" H-2 alone is insufficient to convey disease susceptibility.

Significance to Biomedical Research and the Program of the Institute:

Clinical premature ovarian failure

a. Bone density

It is striking that, based on bone density at the femoral neck, there is a threefold increase in risk of hip fracture in over one-half of our patients with premature ovarian failure. Hip fracture is associated with major morbidity. The dollar cost of hip fracture in the United States alone is more than $8 billion per year. In addition, there are human costs of pain, functional limitation, reduced quality of life, loss of independence, and inability to work. There is evidence that American women in general are experiencing more hip fractures earlier in life. In other words, the age-specific incidence of hip fracture has been increasing during the past three decades. The reasons for this age-specific increase are unknown, but increasingly sedentary life styles likely play a role.

Even simple questions with tremendous public health import about osteoporosis go unanswered. For example, presently we do not know what type of weight bearing exercise is the most effective to increase bone density in young women. Our patients with premature ovarian failure are an ideal group in which to evaluate strategies to increase bone density and reduce risk of hip fracture. Patients with premature ovarian failure experience periods of hypoestrogenism before the disorder is recognized, and hypoestrogenism causes rapid loss of bone mineral density. Estrogen replacement therapy will halt this rapid loss, but alone will not restore lost bone. A safe and effective method to restore bone density for these women is needed. One in 100 American women develop premature ovarian failure.

b. Autoimmune oophoritis

Our findings highlight the fallacy of adding treatments into clinical practice based on anecdotal reports of success, and emphasize the need for controlled studies to evaluate therapies for this condition. Patients with premature ovarian failure often experience spontaneous remission.

Anecdotal reports have suggested that high-dose, long-term prednisone therapy may be useful in treating autoimmune ovarian failure. However, prednisone, when used in high-dose for a long-term, has substantial side effects. These include aseptic necrosis of bone requiring major surgical intervention. Despite this risk, patients with premature ovarian failure are being treated based on this anecdotal evidence. We are aware of one patient with premature ovarian failure who developed aseptic necrosis of bone on high-dose, long-term prednisone therapy administered elsewhere.

To make progress toward a better understanding of human autoimmune oophoritis will require an integrated approach, such as presented here, to combine clinical and basic laboratory investigation with use of appropriate animal models. A major obstacle to progress in autoimmune premature ovarian failure is the lack of an accurate diagnostic method to identify patients who develop ovarian failure by this mechanism. The need is great to define the pathophysiology of autoimmune ovarian failure, to develop accurate tests to identify the pathogenic mechanisms, and to develop specific efficient therapies to reverse these pathogenic mechanisms without the side effects that accompany generalized immunosuppression.

We are narrowing the search for autoimmune oophoritis. Work by previous investigators has demonstrated that ovarian wedge biopsy has a low yield in diagnosing autoimmune oophoritis. Based on our findings it appears that ovarian biopsy targeted at developing antral follicles will also have low yield in diagnosing this condition.

As practicing physicians become aware of our findings through peer review publications and book chapters, unnecessary health care expenditures related to premature ovarian failure should decline, and these funds will become available for other health care needs. Clinicians seeing patients with premature ovarian failure often order antiovarian antibody titers in an effort to diagnose autoimmune premature ovarian failure. Some also order lymphocyte phenotyping to determine if HLA DR3 is present. These tests are expensive, and, based on our findings, add nothing substantive to the evaluation.

Murine post-thymectomy polyglandular autoimmunity

Autoimmune diseases rank among the major medical problems of today's industrialized societies. Women are more susceptible to autoimmune diseases than men, and autoimmune diseases in children can significantly impair normal growth and development. Independent from their medical and social consequences, autoimmune diseases present fundamental questions about the development and regulation of the immune system. What mechanisms permit the immune system to differentiate autoantigen from invading antigen? Murine post-thymectomy polyglandular autoimmunity is particularly appealing as a model to gain insight into this development and regulation. In this model, autoimmunity is induced by a specific perturbation to the immune system, without the need for administration of exogenous antigen.

a. Inciting ovarian antigen

The novel oocyte-specific gene we have identified just might be the gene that produces the inciting ovarian antigen in this murine model. This gene might allow us to develop a sensitive and specific serum marker for human autoimmune ovarian failure. If we detect an analogous human gene, we can employ the analogous human recombinant protein in an assay to detect oocyte-specific human ovarian autoantibodies. There is precedence for this strategy. In murine post-thymectomy autoimmune gastritis the inciting antigen has been identified as the gastric proton pump. Interestingly, antibodies to the gastric proton pump are also a useful marker for autoimmune gastritis in humans. In many models of organ-specific autoimmunity a single protein antigen plays a key role in initiating disease, and dramatic cellular and humoral responses to this antigen develop early in the process.

b. Perturbing the neonatal immune system

We may have uncovered the major pathophysiologic mechanism by which neonatal thymectomy induces polyglandular autoimmunity. Our findings challenge fundamental mechanisms that have been proposed regarding the development of the neonatal immune system. Furthermore, our findings provide a basis for evaluating and treating human autoimmune disorders from the perspective of abnormal neonatal development.

Proposed Course:

Basic research

We will finish sequencing the novel murine oocyte-specific-expression gene that we identified. To identify this gene, we screened a mouse ovarian lambda gt-11 cDNA expression library with high-titer serum from mice with post-thymectomy autoimmune oophoritis. We will use a "knockout strategy" to gain insight into the normal function of this gene. If we can sequence an analogous human gene, we will employ the analogous human recombinant protein to develop an assay for high-titer oocyte-specific autoantibodies in patients with premature ovarian failure.

We will determine if our novel oocyte-specific gene produces the inciting antigen in murine post-thymectomy autoimmune oophoritis. We will do this by expressing this single autoantigen in the thymus using transgenic techniques. If this is indeed the inciting antigen, the expression of this protein in the thymus should induce specific tolerance. This should abrogate the development of autoimmune oophoritis in A/J mice thymectomized as neonates. This same approach has been used previously to confirm that one specific protein, the ß subunit of H/K ATPase (gastric proton pump), is the inciting antigen in post-thymectomy autoimmune gastritis.

We will extend our experiments in murine post-thymectomy polyglandular autoimmunity to further study the development and regulation of the immune system. If neonatal thymectomy induces disease by impairing the development of a Th1 response, disease should not occur if we can cause the shift to the adult Th1 dominant response to occur before the thymectomy is performed on day 4 of life. To test this hypothesis, we will administer IL-12 on day 2 and 3 of life, perform thymectomy on day 4 as usual, then determine if this treatment restores normal Th1:Th2 balance and ameliorates the autoimmune oophoritis despite thymectomy.

We will also extend our experiments into the clinic by examining children with the candidiasis-endocrinopathy syndrome (autoimmune polyglandular failure type 1) for evidence of a similarly predominant TH2 response in peripheral blood lymphocytes. If in these children such an abnormality in the normal TH1:TH2 profile is indeed present, this would raise the possibility of stimulating TH1 function as a means not only to treat the chronic candidiasis, but also to possibly prevent the development of polyglandular failure.

Clinical research

We will continue our controlled trial of alternate day prednisone therapy in the treatment of biopsy-proven autoimmune premature ovarian failure. This protocol tests the hypothesis that a lower risk therapy (alternate-day, lower dose, shorter-term prednisone) will induce remission of autoimmune ovarian failure.

We will begin a randomized controlled trial to evaluate strategies to improve bone density in women with premature ovarian failure. We will also compare programs of intensive home-based weight-bearing exercise specifically designed to maximally load the hips and spine. Change in bone mineral density at the femoral neck measured by dual energy x-ray absorptiometry will be the primary outcome parameter to be compared.

We will investigate vascular endothelial growth factor as an early molecular marker for ovarian follicle luteinization. To develop new therapies for patients with abnormal ovarian follicle function requires an understanding of normal human ovarian function. Surprisingly, little is known about the local factors that effect and regulate human follicle luteinization. Inappropriate luteinization of graafian follicles could be a major pathophysiologic mechanism preventing normal follicle function in many reproductive disorders. Neovascularization is a major histologic component of luteinization, and therefore, vascular endothelial growth factor might be a useful early molecular marker. Thirty healthy women who will be undergoing reversal of surgical sterilization by minilaparotomy will be recruited. They will have their surgery timed to take place within a 4 day window expected to include the luteinizing hormone (LH) surge. The time of the surge will be determined from blood samples collected every 4 hours during the days preceding surgery. At the time of tubal surgery, the dominant ovarian follicle will be excised using microsurgical techniques. The follicle will be snap frozen, sectioned, and prepared with appropriate radio-labelled probes to detect message for vascular endothelial growth factor. Signal intensity in the theca and granulosa cells will be compared in follicles removed before and after the LH surge.

We will investigate a method of ovulation induction for patients with Stein Leventhal syndrome who are resistant to clomiphene citrate therapy. Ovarian follicles contain androgen receptors, and androgens may play a role in follicle atresia. The hyperandrogenism associated with Stein Leventhal syndrome might impair ovarian follicle development through a receptor-mediated mechanism. Flutamide, a non-steroidal anti-androgen, effectively blocks the action of androgen at the receptor level. We will test the hypothesis that blockade of androgen action with flutamide will permit ovulation in women previously resistant to clomiphene citrate alone. We will employ a randomized, double blind, and placebo controlled study design. We will study 50 patients and determine ovulation rates by serial progesterone levels.

In a new area of investigation for this project, we will evaluate fat-suppressed magnetic resonance imaging (MRI) as a non-invasive method to detect lesions of endometriosis in patients with severe invasive endometriosis. Endometriosis is associated with infertility in women, and evidence suggests the presence of endometriosis may interfere with normal ovarian follicle growth and development. While not classified as a malignancy, severe invasive endometriosis can nevertheless invade bowel, bladder, and even lead to ureteral obstruction. Often the disorder involves prolonged medical treatment and repeated surgical interventions. All currently available conservative treatment strategies for endometriosis are, in essence, palliative. An accurate, non-invasive method to detect and monitor endometriosis would facilitate efforts to develop more effective conservative therapies. Twenty patients with severe invasive endometriosis will be recruited. After appropriate preoperative evaluation and MRI examination, patients will undergo surgery as indicated by their individual clinical situation. The size and location of endometrial implants in the surgical field will be noted. The sensitivity, specificity, and positive predictive value for the fat-saturated MRI technique to detect 4 mm lesions will be generated by standard statistical methods.

Protocols:

91-CH-127 Nelson Ovarian follicle function in patients with premature ovarian failure

This screening protocol is the focal point of our clinical and basic research effort on premature ovarian failure. We use this enigmatic disorder as a springboard to gain insight into ovarian function not only by seeing patients, but also by employing animal models and basic molecular biology in collaboration with basic scientists. In so doing, we take full advantage of the unique environment provided by the NIH Clinical Center. The potential for a broad public health impact of such an approach should not be underestimated. We have recruited 51 of the 150 patients approved for this protocol. From this protocol we have learned that one-half of patients with premature ovarian failure have follicles that function intermittently, and based on controlled studies, we have also found that patients with this disorder can undergo remission after treatment with placebo. This emphasizes the need for controlled studies to evaluate treatments for this disorder.

92-CH-223 Nelson Autoimmune premature ovarian failure: a controlled trial of alternate-day prednisone therapy

No therapy for infertile patients with premature ovarian failure has been proven effective by prospective controlled study. Anecdotal reports have suggested that high-dose, long-term prednisone therapy may be useful in treating autoimmune ovarian failure. However, prednisone, when used in high-dose for a long-term has substantial side effects, including aseptic necrosis of bone requiring major surgical intervention. Despite this risk, patients with premature ovarian failure are being treated based on this anecdotal evidence. We are aware of one patient with premature ovarian failure who developed aseptic necrosis of bone on high-dose, long-term prednisone therapy administered elsewhere. This protocol will test the hypothesis that a lower risk therapy (alternate-day, lower dose, shorter-term prednisone) will induce remission of autoimmune ovarian failure. The protocol will use a double-masked, placebo-controlled design. Patients will undergo ovarian biopsy by laparoscopy to confirm the presence of autoimmune oophoritis. Ovarian biopsy carries a mortality risk approximately equivalent to the risk women accept when they decide to undergo surgical sterilization. Successful outcome will be defined as a return of ovulation as determined by weekly serum progesterone levels. The hypothesis that short-term, alternate-day prednisone therapy restores ovulation will be tested with an equality of proportions test comparing the proportion of patients who ovulate during placebo with the proportion of patients who ovulate during prednisone therapy.

Nelson Use of flutamide in clomiphene resistant anovulation

Anovulation associated with polycystic ovary syndrome causes infertility. Clomiphene citrate is the first line treatment to induce ovulation in these women because it is simple and effective therapy. However, in some cases clomiphene fails to induce ovulation. Presently, we have no simple and effective therapy for anovulatory women with polycystic ovaries who fail to respond to clomiphene citrate.

This protocol will test the hypothesis that the hyperandrogenism associated with polycystic ovary syndrome impairs follicular development by a receptor-mediated mechanism. Ovarian follicles contain androgen receptors, and androgens are thought to play a role in ovarian follicle atresia. Flutamide, a non-steroidal anti-androgen, effectively blocks the action of androgen at the receptor level. Therefore, we will test the hypothesis that flutamide, given along with clomiphene citrate, will permit clomiphene-resistant patients to ovulate. We will employ a randomized, double-blind, placebo-controlled study design. We will study 50 patients and determine ovulation rates by serial progesterone levels. If we assume a 10% spontaneous ovulatory rate, and we seek to detect an ovulation rate of 35% in the treatment group, we have the power to detect this difference 92% of the time.

93-039	Nelson	Immunogenetics of autoimmune oophoritis in the neonatally thymectomized mouse model
93-032	Nelson	Autoimmune ovarian failure (murine)

Publications

Moy E, Kimzey LM, Nelson LM, Blithe DL. Glycoprotein hormone α-subunit functions synergistically with progesterone to stimulate differentiation of cultured human endometrial stromal cells to decidualized cells: a novel role for free α-subunit in reproduction. Endocrinol 1996;137:1332-9.

Nair S, Caspi RR, Nelson LM. Susceptibility to murine experimental autoimmune oophoritis is associated with genes outside the major histocompatibility complex. Am J Reprod Immunol, in press.

Nair S, Mastorakos G, Raj S, Nelson LM. Murine experimental autoimmune oophoritis develops independently of gonadotropin stimulation and is primarily localized in the stroma and theca. Am J Reprod Immunol 1995;34:132-9.

Nelson LM, Anasti JN, Flack MR. Premature ovarian failure. In: Reproductive endocrinology, surgery, and technology. Adashi EY, Rock JA, Rosenwaks Z (eds). New York, Raven Press 1995;1393-410.

Raj S, Nair S, Mastorakos G, Nelson LM: Anamnestic development of lymphocytic infiltration in murine experimental autoimmune oophoritis is primarily localized in the stroma and theca. Am J Reprod Immunol 1995;34:125-31.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00636-04 DEB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Endocrinology of Reproduction in Women

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	James H. Segars	Sr. Clinical Investigator	UMMR, SGR, DEB, NICHD
Others:	Paul Driggers	Chemist	UMMR, SGR, DEB, NICHD
	Domenica Rubino	Clinical Associate	UMMR, SGR, DEB, NICHD
	Lawrence Kemp	Pre–IRTA Fellow	UMMR, SGR, DEB, NICHD
	Susan Nunez	Clinical Associate	UMMR, SGR, DEB, NICHD

COOPERATING UNITS *(if any)*

K. Ozato, SMGI, Laboratory of Molecular Growth Regulation, NICHD;
L. Nieman, URM, Section on Womens Health, Developmental Endocrinology Branch, NICHD

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Womens Health, Unit on Molecular Mechanism of Reproduction

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The broad objective of this project is to gain further understanding of hormonal mechanisms governing reproductive processes in women which will provide the foundation for clinical therapies directed toward reproductive disorders such as endometriosis, pregnancy loss, fertility, and estrogen-dependent reproductive neoplasms. We have approached this objective by the study of proteins responsible for mediating and/or modulating estrogen-dependent reproductive processes. Advances during the current period include: 1) sequencing and initial characterization of a novel estrogen receptor binding protein; 2) demonstration that the novel gene is expressed in hormonally responsive reproductive tissues such as ovary, breast, and endometrium; 3) demonstration that the novel protein interacts specifically with the estrogen receptor *in vitro*; 4) demonstration that overexpression of the protein markedly influences estrogen receptor function; 5) demonstration that interaction between the novel protein and the estrogen receptor requires discrete regions of the estrogen receptor as well as distinct regions of the novel receptor-binding protein. Given the central role of estrogen in reproduction, we plan to continue to concentrate effort on delineation of the possible role of this gene in the regulation of hormonally dependent reproductive processes.

Project Description:

Objective: The overall objective remains unchanged during the current period. The broad objective is to understand the mechanisms involved in tissue-specific regulation of nuclear hormone receptor function, particularly in reproductive tissues. Due to the central role of estrogen in reproduction, we have concentrated on proteins and receptors capable of affecting estrogen-dependent responses. As was proposed, during the past period we have focused on the study and characterization of the novel nuclear hormone receptor binding protein isolated by our group (see prior reports). Specifically, we have: 1) characterized the regions and domains of the novel protein with regard to interaction with nuclear receptors, particularly the estrogen receptor; 2) tested the retinoid X responsive and estrogen-responsive genes for regulation by the novel gene; and 3) studied the tissue distribution of the novel gene.

Methods:

Standard and automated fluorescent sequencing methods were used to determine the sequence of plasmid constructs and cDNA fragments. Epitope-tagged expression plasmids (GST and FLAG) were used for expression of recombinant protein in bacteria and mammalian cells. Dual hybrid yeast system was used to test for interaction between proteins. Cotransfection assays using luciferase-based reporters standardized with expression of galactosidase was used to evaluate function of the protein *in vivo*.

In vitro interaction between receptors and proteins was studied using co-immunoprecipitation, farwestern, and GST-binding studies. In addition, receptors and associated proteins were studied with standard western, northern, Southern, and mobility shift analysis.

Nonradioactive detection methods such as chemiluminescence were used in western and immunohistochemistry detection of the novel protein.

Major Findings:

During the current period we used serial screening of cDNA libraries to isolate multiple overlapping cDNA clones. In addition, reverse transcriptase polymerase chain reactions (RT-PCR) were used to confirm 5' cDNA ends. Sequencing of the multiple overlapping clones and RT-PCR products generated a cDNA encoding a novel receptor-binding protein of a size consistent with mRNA detected using northern analysis. Initially isolated based on binding to the retinoid X receptor (RXR), further studies suggested avid binding of the novel protein to the estrogen receptor (see below). Since the protein representated a **b**reast cDNA **r**eceptor-binding au**x**iliary protein, we called the protein **brx**.

We observed the gene brx to be expressed predominantly, but not exclusively, in reproductive tissues. Specifically, the gene was abundantly expressed in breast, ovary, testes, and endometrial tissues. Localization of brx message was noted in hormonally-responsive cells known to contain the estrogen receptor, thus suggesting that brx might play a role in the regulation of estrogen-mediated responses.

In a series of *in vitro* studies using bacterially expressed brx and *in vitro* expressed labeled estrogen receptor, we demonstrated that brx bound specifically to the estrogen receptor. Converse experiments were used to confirm binding of the estrogen receptor to brx. Further, antibody-meditated precipitation experiments were used to confirm the specificity of interaction. Together, these experiments suggested that discrete regions in the carboxyl region of the estrogen receptor bound to distinct regions in brx.

Of particular significance, we performed cotransfection assays in a human endometrial cell line to demonstrate that overexpression of brx markedly augmented estrogen-dependent activation of reporter constructs containing estrogen receptor DNA target sequences. The observed activation was specific for estrogen receptor-mediated gene activation and was not attributable to trivial, artifactual, or non-specific effects on the reporter system. Specificity of activation was further confirmed using a series of brx deletion mutants in the cotransfection assays. This series of experiments suggested that brx might be involved in estrogen-dependent gene regulation. To test whether brx might localize to the nucleus and confirm expression of brx in the endometrial cells, we studied localization of epitope-tagged brx using immunohistochemistry. Signal attributable to brx was observed to be present in the nucleus, consistent with a role in estrogen-mediated responses.

Significance to Biomedical Research and the Program of the Institute:

Reproductive health is a major focus of the NICHD. A fundamental characteristic of reproductive tissues in women is the ability of the tissues to respond to the hormone estrogen. Estrogen transmits signals to reproductive tissues through both nongenomic and genomic effects. Genomic effects of estrogen are best understood and are mediated by estrogen receptor action through target DNA sequences. Despite advances in understanding of estrogen receptor action, the mechanisms governing tissue-specific regulation of estrogen receptor function remain unclear. A detailed understanding of tissue-specific regulation of nuclear receptor function is of clinical importance. Such mechanisms are believed to explain the observation that tamoxifen, for instance, acts as an anti-estrogen in breast tissue, but functions as a estrogen agonist in endometrium. This paradoxical action of tamoxifen results in abnormal growth of the endometrium in patients treated with tamoxifen for treatment of breast cancer. At present, it is believed that such tissue-dependent effects of estrogen receptor function are conferred by receptor-associated proteins that govern estrogen receptor activation. Given our observation that brx augments estrogen receptor mediated gene activation, brx is a prime candidate for a gene that might regulate tissue-specific function of the estrogen receptor.

There are two additional observations regarding brx that suggest the gene may be of importance in estrogen-mediated responses. First, in contrast to most nuclear receptor-associated proteins reported to date which are widely expressed, brx is predominately expressed in reproductive tissues. In particular, brx is present in endometrial tissue. Second, expression of brx closely matched expression of the estrogen receptor. Taken together, these observations argue for further study of brx as a protein capable of influencing estrogen receptor action.

As suggested above, detailed understanding of estrogen receptor action may contribute to understanding of reproductive disorders. For example, endometriosis represents a condition where the lining of the uterus acquires the ability to grow and proliferate in abonrmal locations in women. Endometriosis is a common reproductive disorder that contributes to infertility, pelvic pain, loss from work, and ultimately hysterectomy in some cases. Of interest, expression of estrogen and progesterone receptors is noted to be abnormal in a substantial proportion of endometriotic tissues. The abnormal expression of estrogen and progesterone receptors is suspected to contribute to the resistance of endometriotic tissue to hormonal therapy. While the mechanism remains unclear, it is suspected that function of nuclear receptors is impaired in endometriotic tissues. Thus, a detailed understanding of estrogen receptor function may have broad implications for common reproductive disorders such as endometriosis. The specific role of brx as a putative regulator of estrogen receptor function remains to be defined, yet is of interest since present understanding of nuclear receptor function in disorders such as endometriosis is limited. Further studies of brx in endometriotic tissues are required to test the possible involvement of brx in this, or other reproductive disorders.

Proposed Course:

We plan to continue to study the relationship of brx to estrogen receptor function. The proposed studies will attempt to define the precise role of brx in estrogen receptor-mediated responses. To accomplish this aim, we plan to generate antibodies directed against brx, and to use this tool to analyse estrogen-responsive tissues. Studies will be focused on the specific mechanism by which brx affects estrogen receptor action, particularly *in vivo*. In addition, we plan to express brx using baculovirus expression system inorder to obtain partially purified protein for *in vitro* studies.

We hope to continue study of endometrium, both normal and abnormally implanted tissue, inorder to determine whether brx may have a role in disorders such as endometriosis. If initial studies are promising, we plan to explore the possible interaction of brx with other nuclear receptors.

Publications:

Leong G, Marton M, Wang K, Blanco JCG, Wang IM, Rolfes R, Ozato K, Segars JH. In vivo interaction between RXR and TFIIB requires 9-cis retinoic acid and the AF-2 region of RXR. Mol Cell Biol, in press???

Nunez SB, Medin JA, Braissant O, Wahli W, Ozato K, Segars JH. The retinoid X receptor and perosisome proliferator-activated receptor activate estrogen responsive genes independent of the estrogen receptor. Mol Cell Endocrinol, in press.

Segars JH. Endometriosis and nuclear hormone receptors. In: Diamond M, Osteen K, eds. Endometrium and Endometriosis. Cambridge: Blackwell Science, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD-00637-03 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Steroid Hormone Action in Female Reproduction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	L.K. Nieman	Senior Clinical Investigator	URM, SWH, DEB, NICHD
Others:	J. Segars	Sr. Clinical Investigator	MMTP, SWH, DEB, NICHD
	T. Kim	Special Volunteer	URM, SWH, DEB, NICHD
	P. Driggers	Chemist	MMTP, SWH, DEB, NICHD
	M. Passaro	Clinical Associate	URM, SWH, DEB, NICHD
	K. Weichold	Visiting Fellow	URM, SWH, DEB, NICHD

COOPERATING UNITS *(if any)*

Maria Merino, M.D., Clinical Pathology, NICHD; Nancy Alexander, Ph.D., Contraceptive Development Branch, NICHD; Asgi T. Fazleabas, Ph.D., University of Illinois at Chicago

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Womens Health, Unit on Reproductive Medicine

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to understand better the development of the endometrium and oocyte in women and to investigate the role of gonadal steroids in these processes.

The process of uterine remodeling that is so remarkable in the mammalian uterus is achieved through coordinated proliferation, differentiation and cell death. While the gonadal steroids estradiol and progesterone appear to be required for these organ-specific processes, the mechanism(s) by which these processes are regulated remain unclear. Potential mediators of estrogen action include IGF-1 and c-Myc, a transcription factor known to be induced by growth factors. We used a well-characterized in vivo ovariectomized mouse model to correlate sex steroid-induced proliferation with the induction of IGF-1 and c-Myc mRNA in the uterine endometrium. We have found that IGF-1 and c-Myc levels parallel each other and the estrogen-induced processes of differentiation in this model, suggesting that they are important to proliferation. The induction of IGF-1 by GH in other tissues prompted an evaluation of whether the GH receptor was present in the endometrium, based on the hypothesis that GH might act directly on the endometrium and so modulate estrogen effect. Previous findings that the GH receptor was present throughout the endometrium, also showed that GH was present in the myometrium. Further studies revealed that GH receptor was greater in leiomyomas than in surrounding myometrium, suggesting that GH reduction by octreotide might be a novel therapeutic approach to these lesions.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

Our long-term goal is to investigate infertility, to develop new contraceptives, and to evaluate new approaches to therapy of the menopause. Recent studies have sought to understand better the role of growth factors in endometrial proliferation and differentiation.

The process of uterine remodeling that is so remarkable in the mammalian uterus is achieved through coordinated proliferation, differentiation and cell death. While the gonadal steroids estradiol and progesterone are required for these organ-specific processes, the mechanism(s) by which these processes are regulated remain unclear. We postulate that IGF-1 and c-myc mediate estrogen's mitogenic effects and that growth hormone (GH) as well as estrogen might induce endometrial IGF-1 and mitogenesis. Observations that growth hormone increases uterine weight independent of estradiol in hypophysectomized rats and is associated with an increased incidence of fibroids in acromegalic women support this idea. Our previous findings that GH receptor is present in estrogen treated murine uterus, suggested that GH may play a role in the development of fibroid tumors from women.

It is known that progesterone is necessary for endometrial differentiation, but the specific progesterone-dependent peptides critical to this process have not been characterized. The dyad of hepatocyte growth factor (HGF) and its receptor, MET, may mediate progesterone-induced epithelial differentiation. In other tissues, such as breast, lung and liver, HGF production by mesenchymal stroma appears to direct tubular differentiation via its epithelial expressed receptor, MET. By analogy, we hypothesize that a similar paracrine relationship exists between uterine epithelial and stromal compartments, so that MET and/or HGF are induced by progesterone and influence glandular differentiation of the epithelium. To our knowledge, HGF has not been localized to human endometrium. While the promoter region of MET has been cloned, little is known about tissue-specific regulation of this gene. Thus, we have also examined whether sex steroids, phorbol esters, or HGF, alter transcription from the MET promoter.

Methods Employed:

GH receptor in leiomyoma: Leiomyomata and surrounding myometria were obtained at the time of surgery from women who did or did not receive preoperative GnRHa therapy. Tissues were processed for mRNA extraction and PCR detection of the GH receptor and GAPDH, and for in situ hybridization.

HGF and MET system in human endometrium: Endometrial biopsies were obtained from normally cycling women and dated according to Noyes' criteria. Tissue was fixed, and stained for HGF or MET using biotin-avidin colorimetric detection of antibody. Positive controls were 184B5 breast epithelium (MET) and WI 38 lung fibroblast (HGF).

Animal studies: An oophorectomized murine model was used to examine whether IGF-1 and c-myc might mediate gonadal steroid action in the uterine glands and stroma. In situ hybridization for IGF-1 and c-myc mRNA was correlated with mitotic index after direct Alzet pump infusion of IGF-1 into the cornua, or after subcutaneous administration of RU 486, progesterone and/or estrogen.

Promoter studies: Ishikawa human endometrial adenocarcinoma cells were grown to confluence in D-MEM/Ham's F-12 with 5% charcoal-stripped fetal bovine serum. Cells were transfected with a reporter gene construct containing either 400 or 2700 base pairs upstream of the MET gene transcriptional start site fused to pGL2-Basic Vector or a B-galactosidase control expression vector. Luciferase activity was compared after treatment with HGF, phorbol ester, estrogen and/or progesterone. The pGL2-Basic vector lacking the MET promoter was used as a control.

Major Findings:

Presence of GH receptor in leiomyoma: GH receptor mRNA was present in both leiomyoma and surrounding myometrium with greater expression in leiomyoma, suggesting that GH may act directly on the human uterus. GnRHa treatment reduced GH receptor gene expression, implying that GH receptor expression in leiomyoma may be regulated by sex steroids.

HGF and MET expression in human endometrium: Preliminary data (Rarick T., abstract) support our hypothesis that HGF is produced by stromal cells, and that the receptor is present in epithelial cells. Data from two follicular phase specimens show stromal HGF staining, without MET; luteal phase specimens show both stromal HGF and epithelial MET. This temporal relationship parallels the increase in plasma progesterone in the luteal phase, and suggests that MET may be regulated, directly or indirectly, by progesterone.

Animal studies: After gonadal steroid treatment, endometrial IGF-1 and c-myc mRNA expression increased, and paralleled the pattern of differentiation and proliferation. Treatment with estrogen (alone and with progesterone and RU 486) induced proliferation of the epithelial cells, while the addition of progesterone shifted mitosis to the stroma and resulted in differentiation of the epithelium. IGF-1 and c-myc showed coordinate expression in the epithelium (estrogen treatment) or both compartments (estrogen and progesterone treatment). The c-myc expression after IGF-1 alone was similar to that seen with estrogen. These preliminary findings suggest a role for IGF-1 as an estromedin.

Promoter studies: HGF and phorbol ester enhanced activity of the MET promoter by 10-fold or more. This effect was additive. In contrast, neither estrogen nor progesterone altered activity of the promoter, suggesting that in vivo induction of MET observed in the luteal phase (above), is not directly mediated by progesterone.

Significance to Biomedical Research and the Program of the Institute:

These studies reflect the NICHD mission to understand better the physiology of normal and abnormal reproductive states and to develop contraceptive agents. The ability of the uterus to undergo coordinated timely growth and regression provides an excellent model to understand the influence of growth factors and protooncogenes on cell cycle progression and the way in which hyperplasia and uncontrolled cell growth is prevented in vivo.

Proposed Course:

1. Characterization of MET and HGF in the endometrium

Two experimental models will be used: in vitro cell culture and examination of endometrial biopsies obtained from normally cycling women.

In vitro Cell Culture:

We will continue to use the well-characterized estrogen-responsive human endometrial adenocarcinoma cell line, Ishikawa Var 1, as a model for epithelial cells. Cells will be grown to confluence in DMEM/Hams F12 (1:1) media with 5% fetal calf serum. To study the effects of steroids, charcoal-stripped serum and phenol-free media will be substituted for 72 hours, followed by the addition of physiologic amounts of estradiol (150 pg/ml) and/or progesterone (0 - 40 pg/mL) for up to seven days. Cells will be harvested and processed for immunohistochemistry for MET. A dose-response curve of protein expression in response to progesterone will be generated. Antiprogestin (RU 486 or CDB 2914, 10 pg/mL) will be added, to confirm that peptide induction depends on the progesterone receptor.

It is possible that stromal cell HGF production mediates progesterone effects on epithelial cell differentiation, as suggested by the preliminary results with the MET promoter (above). To examine this, synthetic HGF will be added to culture media with or without estrogen and or progesterone. Ishikawa cells will be harvested and examined for MET expression by immunohistochemistry.

If this Ishikawa cell model fails to reveal induction of MET, we will perform short-term co-culture studies of human epithelial and stromal endometrial cells. Late follicular phase or early luteal phase biopsies will be obtained and epithelial and stromal cells will be separated by sieving using the method of Osteen. The cell preparations will be checked for purity using vimentin and cytokeratin immunohistochemistry. Gland and stroma-enriched isolates will be cultured alone or together with or without hormones as described above. Epithelial components will be grown in matrigel well inserts to promote polarized, anatomically correct growth and to prevent direct stromal cell contact, so that only diffusible stromal factors will influence the epithelial cells. Epithelial cells will be harvested at intervals and examined for the presence of MET. Controls from other fibroblast cell lines, such as dermal fibroblasts, will be used to assess the specificity of endometrial stromal cell effect.

Human Studies: Endometrial Biopsies for Culture and Immunohistochemistry or In Situ Hybridization

Normally cycling women will be recruited to undergo an endometrial biopsy, blood sampling and ovarian ultrasound. All cycles will be characterized hormonally by determination of the LH surge and by measurement of estradiol and progesterone. Follicular phase biopsies will be correlated with the size of the dominant follicle by ultrasound. Luteal phase biopsies will be timed according to the LH surge and will be obtained using a Pipelle curette. A part of each specimen will be placed in formalin for dating by Noyes' criteria. The remaining tissue will be used for study procedures, including tissue culture (as above), or in situ hybridization/immunohistochemistry to localize mRNA or peptides to epithelial and/or stromal cells and to correlate changing patterns of expression over the menstrual cycle with hormone levels. The DNA sequence of the candidate peptides is known, and standards and antibodies to each are in use in our laboratory or available to us. This will allow us to confirm and extend our preliminary observations about the HGF/MET system across the menstrual cycle.

2. Clinical uses of antiprogestins: We previously showed that RU 486 can retard endometrial maturation in women and inhibit implantation in the guinea pig, suggesting that antiprogestins may be effective contraceptive agents that render the endometrium hostile to implantation without affecting hormonal rhythm. Further development of these properties of antiprogestins would represent a significant new advance in contraceptive technology. Additionally, data from two groups suggests that RU 486 has an "anti-estrogenic" effect on the endometrium: when given to ovariectomized, estrogen-replaced rhesus monkeys, the compound causes endometrial atrophy. This property of antiprogestins, if replicated in post-menopausal women, might have significant implications for development of estrogen-antiprogestin combination therapy of the menopause.

RU 486 is no longer available from Roussel-UCLAF. However, the Contraceptive Development Branch, NICHD, has another antiprogestin, CDB 2914. Results from pre-clinical studies suggest that its activity is similar to that of RU 486. We will develop this compound for extramural and intramural clinical trials, beginning with protocol 95-CH-0168, "Dose-response Relationships for the Antiprogestin CDB 2914 in Cycling Women". Women will receive the compound in the luteal phase in this phase 1-2 trial, to determine safety and to provide initial information about biologic effects. Blood will be obtained for pharmacokinetic studies, and for evaluation of changes in cortisol, ACTH, LH, FSH, estradiol and progesterone. Women will chart menses and basal body temperature to evaluate antagonism of endometrial or hypothalamic progesterone action. Should the compound appear promising, another protocol will be proposed to include endometrial biopsy, which will provide placebo or antiprogestin-exposed tissue to test whether MET/HGF are regulated by progesterone.

Studies in rhesus monkeys: In collaboration with Dr. Fazleabas, rhesus monkeys will be ovariectomized and receive vehicle, estrogen alone, estrogen with progesterone, or estrogen with CDB 2914 at a small daily dose. Endometria will be biopsied and examined for hormonal effects on proliferation, to determine whether CDB 2914 has an anti-estrogen effect.

Publications:

1. Sharara FI & Nieman LK. Growth hormone receptor mRNA expression in leiomyoma and surrounding myometrium. Am J Obstet Gynecol 1995;172:814-9.

Patents:

none

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00638-03 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Physiology of Hypercortisolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	L.K. Nieman	Research Medical Officer	URM, SWH, DEB, NICHD
Others:	J. Yanovski	Supervisory Medical Officer, Pediatrics	SDE, DEB, NICHD
	D. Papanicolaou	Clinical Associate	SPE, DEB, NICHD
	G. Chrousos	Chief, SPE, DEB, NICHD	SPE, DEB, NICHD
	G. Cutler, Jr.	Chief, SDE, DEB, NICHD	SDE, DEB, NICHD

COOPERATING UNITS *(if any)*

John Doppman, Department of Radiology, CC; Edward Oldfield, SND, NINDS; Konstantine Kalogares, Clinical Neuroendocrinology Branch, NIMH

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Womens Health, Unit on Reproductive Medicine

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cushing syndrome (CS), a fatal disease, is suspected in many thousands of patients each year, but confirmed in only a fraction of these. This project seeks to identify accurately which patients have Cushing syndrome, to define the etiology of their disease, and to treat it optimally.

A major initiative in the past year has been to examine the potential role of vasopressin in the pathogenesis of Cushing's disease. Vasopressin (VP) is known to induce ACTH secretion, and thus might stimulate corticotropes to form tumors. We measured vasopressin concentrations in the petrosal sinuses of patients with Cushing's syndrome and normal volunteers. Unexpectedly, regardless of diagnostic group, nearly all subjects showed a "dominant" petrosal sinus on basal sampling, so that one of the petrosal sinuses had greater levels of both ACTH and VP than the contralateral sinus. However, the absolute concentration of VP was greater in patients with CS than in normal volunteers, and was similar in patients with various causes of CS. Petrosal sinus VP levels in the dominant petrosal sinus increased further after CRH administration. The similarity of petrosal VP levels in CS suggests that VP is not involved in the etiology of Cushing's disease.

Other studies during this reporting period focussed on diagnostic and treatment approaches for patients with adrenal masses. We examined the role of surgery in management of pheochromocytoma, demonstrating that bilateral adrenalectomy should not be performed in the setting of ACTH secretion from the tumor.

Project Description

Objectives:

This project seeks to identify accurately which patients have Cushing's syndrome, to define the etiology of their disease, and to treat it optimally. Cushing's syndrome, a fatal disease, is suspected in many thousands of patients each year, but confirmed in only a fraction of these. Once identified, its cause must be found and successfully treated. The identification, differential diagnosis and treatment of this syndrome pose problems for the clinician. Similarly, the etiology of the causes of Cushing's syndrome remains obscure in the vast majority of cases.

One hypothesis regarding the etiology of Cushing's disease is that hypothalamic or other factors stimulate corticotrope cells to proliferate and form tumors. One candidate for this role is vasopressin, which is known to stimulate ACTH release from corticotropes. The presence of elevated vasopressin levels in patients with Cushing's disease as compared to normal individuals would support the hypothesis that vasopressin plays an etiologic role in corticotrope tumorigenesis. We thus compared levels of vasopressin and ACTH in petrosal sinus blood from patients with Cushing's syndrome of various etiologies, and normal volunteers.

There is no test that reliably separates individuals with pseudo-Cushing's states, who have mild hypercortisolism and minimal physical features of Cushing's syndrome, from those with mild or intermittent Cushing's syndrome, who may present in an identical fashion. We are exploiting the different pathophysiology in the pseudo-Cushing's and Cushing's syndrome states to develop new tests for this distinction. Patients with Cushing's syndrome have either an ACTH-dependent abnormality (corticotrope adenoma or ectopic ACTH-secreting tumor), or a primary adrenal tumor; in this setting the CRH neuron is appropriately suppressed. In contrast, patients with pseudo-Cushing's syndrome have an hypothalamic abnormality, hypersecretion of CRH; the pituitary and adrenal glands are normal. As a result of these differences, we hypothesize that patients with pseudo-Cushing's syndrome might maintain a normal diurnal pattern of cortisol secretion, in distinction to the invariant cortisol levels seen in patients with Cushing's syndrome.

Factitious Cushing's syndrome from surreptitious self-administration of glucocorticoids is a known cause of Cushing's syndrome that may be particularly difficult to identify. We retrospectively reviewed our experience with this condition to establish its prevalence in a large population of tertiary referral patients, and to identify any characteristics that would facilitate this identification.

Other initiatives focussed on the diagnostic and surgical approaches to patients with adrenal masses. While most primary adrenal hyperfunction affects only one adrenal gland, often patients present with non-functioning masses on the contralateral gland on structural scans, confounding the diagnosis of a unilateral disease process. Similarly, some bilateral processes present with only a unilateral mass on structural scanning. We evaluated the utility of ACTH measurement and iodocholesterol adrenal scanning to clarify the diagnosis, and thus to indicate whether unilateral or bilateral resection is appropriate.

Methods Employed:

The methods used in these clinical investigations involve admission of normal volunteers or patients with suspected hypercortisolism. Standard blood and urine collection for hormone measurements by RIA with and without administration of a variety of provocative agents were performed. Additionally research studies (measurement of vasopressin and ACTH in petrosal sinus samples of normal volunteers and patients with Cushing's syndrome, measurement of salivary cortisol) were performed. Standard radiographic studies and catheterization procedures were performed to identify a tumor. Standard iodocholesterol scanning was evaluated as an adjunctive diagnostic tool. The diagnosis of pseudo-Cushing's syndrome is confirmed by long-term physical and biochemical evaluation that fails to demonstrate convincing evidence for evolving Cushing's syndrome. The diagnosis of Cushing's syndrome is confirmed by physical examination and standard biochemical criteria; an etiologic diagnosis is assigned only after surgical cure or histopathologic identification of an ACTH-containing or primary adrenal tumor.

Major Findings:

1. Vasopressin (VP) levels in the petrosal sinuses. Nearly all subjects, both patients with Cushing's syndrome and normal volunteers, showed a "dominant" petrosal sinus on basal sampling, so that one of the petrosal sinuses had greater levels of both ACTH and VP than the contralateral sinus. However, the absolute concentration of VP was greater in patients with Cushing's syndrome than in normal volunteers. VP levels in the dominant petrosal sinus increased further after CRH administration in normal individuals, and in all patients with Cushing's syndrome. VP levels also increased significantly after CRH in the contralateral (non-dominant) petrosal sinuses in patients with Cushing's disease and adrenal disease, but not normal volunteers or those with ectopic ACTH secretion. The finding of a "dominant" petrosal sinus and stimulation of petrosal VP values by CRH are new findings in humans. The similarity of petrosal VP levels in all patients with Cushing's syndrome suggests that VP is not involved in the etiology of Cushing's disease. Furthermore, the increased petrosal VP levels, and the greater responses to CRH seen in Cushing's syndrome suggest that the lack of endogenous CRH stimulation leads to a greater CRH-releasable pool of VP.

2. Factitious Cushing's syndrome. We examined retrospectively 860 charts of patients evaluated for Cushing's syndrome to report our experience with factitious causes. We reported six patients with Cushing's syndrome caused by surreptitious use of exogenous steroids. All had previous unrelated surgical procedures and a history of depression or anxiety. Four had contact with the medical profession. The physical and biochemical features were variable. The most helpful laboratory test was measurement of synthetic glucocorticoids by HPLC. Thus, we recommend such measurements when this diagnosis is suspected.

3. Diagnostic and therapeutic approaches to adrenal masses. We examined the role of surgery in management of pheochromocytoma, demonstrating that bilateral adrenalectomy should not be performed when these tumors secrete ACTH.

Significance to Biomedical Research and the Program of the Institute:

An improved understanding of the etiology of Cushing's syndrome has potential to guide new diagnostic and therapeutic approaches. The emphasis of this program on improved diagnosis and treatment has great potential impact on public health. In our experience, the greatest challenge in the evaluation of patients with possible Cushing's syndrome is to establish the diagnosis. Cushing's syndrome is considered, but is statistically improbable, in thousands of patients each year. The currently available tests do not minimize equivocal information and may lead to a mistaken diagnosis of Cushing's syndrome, which prompts further tests and places the patient at risk for inappropriate intervention, while increasing health care expenditure.

In contrast, our efforts to refine the approach to the differential diagnosis of Cushing's syndrome has a smaller overall benefit to the public health. However, we are now recognized as one of a handful of centers in the world with experience of more than 200 cases, and so we have a unique opportunity to develop an accurate, cost-effective diagnostic strategy that can be used by others with less experience. Our ongoing analysis of a large group of patients should enable us to simplify, standardize and set criteria for the interpretation of these tests. Also, by quickly evaluating reports advocating new tests based on small numbers of patients, we help to establish whether the new tests have value in a larger population. One concrete result of our efforts is the recent FDA approval of human CRH as a diagnostic agent for Cushing's syndrome. This advance was bolstered substantially by work done through this project

Proposed Course:

Diagnosis of Cushing's syndrome. We will continue to measure diurnal plasma cortisol to assess its power to discriminate between pseudo-Cushing's syndrome and Cushing's syndrome. Preliminary data indicate that this test has a high diagnostic accuracy. Using a criterion of serum cortisol of 7.5 ug/dL or more at midnight, 96% patients with Cushing's syndrome were detected and no normal individuals or those with pseudo-Cushing's syndrome were mis-diagnosed. However, as this test is not practical outside of the research setting, we are obtaining salivary cortisol measurements and comparing this test with the plasma test. We anticipate this change in methodology may improve the test's sensitivity, as hypercortisolism suppresses CBG and increases free cortisol, the fraction measured in saliva. It also will make the test convenient and feasible for in-home use, further decreasing the cost of evaluation for Cushing's syndrome.

Differential Diagnosis of Cushing's syndrome. The availability of CRH and the development of IPSS and CRH testing has revolutionized the differential diagnosis of Cushing's syndrome, leading to near 100% sensitivity and specificity in our hands. It is unlikely, however, that IPSS will become widely available, since significant catheterization experience is necessary for its success. Additionally, it has a small, but measurable morbidity, and there is no consensus as to its appropriate role in the diagnostic strategy for Cushing's syndrome. We continue, therefore, to analyze the performance of all tests, with the goal of developing a simple and convenient decision tree for the evaluation of Cushing's syndrome that minimizes cost without compromising diagnostic accuracy.

Because inferior petrosal sinus sampling is dependent on the skill of the radiologist, it is not widely available. Additionally, the morbidity of cerebrovascular accident attendant to the procedure is probably related to occlusion of small vessels. For these reasons, we are interested in exploring the diagnostic utility of jugular venous sampling for measurement of CRH-stimulated ACTH levels. We hypothesize that this procedure may have a diagnostic accuracy similar to that of inferior petrosal sinus sampling. It would have the advantage of being easier to perform, thus more widely available, and might also be more safe. We plan to compare the two procedures in 30 patients.

Unfortunately, the localization of ectopic ACTH-secreting tumors fails initially in 50% of these patients, and may not be achieved for 20 years, exposing them to the deleterious effects of hypercortisolism and the risk of metastatic disease. Thus, we plan to focus on promising venues for the localization of these tumors. We will evaluate the utility of radionuclide-labeled octreotide, a somatostatin analog, for the detection of ectopic ACTH-secreting tumors, and will explore the feasibility of total body PET scan for identification of intrathoracic lesions.

The rate of recurrence in patients successfully "cured" of Cushing's disease by transsphenoidal surgery has not been established, but may be as great as 10%. We continue to follow our cohort to determine the risk of recurrence in the group and are extending our earlier observation that the response to CRH in the post-operative period may predict those patients destined to recur. Should this be clearly established in a large group of patients, strong consideration may be given to early x-irradiation therapy in patients with abnormal post-operative CRH responses.

Publications:

Friedman TC, Nieman LK, Yanovski JA, Doppman JL, Cutler GB, Oldfield EH, Chrousos GP, Kalogeras DT. Inferior petrosal sinus arginine vasopressin concentrations in normal volunteers and patients with Cushing disease. J Clin Endocrinol Metab, in press.

Kalogeras KT, Nieman LK, Friedman TC, Doppman JL, Cutler GB Jr., Chrousos GP, Wilder RL, Gold PW, Yanovski JA. Inferior petrosal sinus sampling in healthy human subjects reveals a unilateral corticotropin-releasing hormone-induced argininine vasopressin release associated with ipsilateral adrenocorticotropin secretion. J Clin Invest 1996;97:2045-50.

Nieman LK. Cushing's Syndrome Treatment. Current Therapy of Endocrinology and Metabolism, C. Wayne Bardin MD, Ed., 1997 edition, in press.

Nieman LK. Treatment of Cushing's Syndrome. Conn's Current Therapy, 1997 edition, in press.

Yanovski JA, Friedman TC, Nieman LK, Doppman JL, Cutler GB Jr, Chrousos GP, Kalogeras KT. Inferior petrosal sinus arginine vasopressin in patients with Cushing syndrome. Clin Endocrinol, in press.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00639-01 DEB

PERIOD COVERED

January 1 1996 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetics of Diabetes Mellitus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Accili, M.D.	Senior Clinical Investigator	UGHA, DEB, NICHD
Others:	G. Di Cola, M.D.	Visiting Fellow	UGHA, DEB, NICHD
	K. Rother, M.D.	Clinical Associate	UGHA, DEB, NICHD
	M. Cool	Biologist	UGHA, DEB, NICHD
	C. Hanash	Pre-IRTA Fellow	UGHA, DEB, NICHD

COOPERATING UNITS *(if any)*

Joslin Diabetes Center, Harvard Medical School (C.R. Kahn, J. Bruening)
Department of Genetics, Columbia University (A. Efstratiadis, A. Louvi)
Department of Physiology, Bar Ilan University, Israel (S. Sampson)

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Unit on Genetics and Hormone Action

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Non-insulin-dependent diabetes mellitus (NIDDM) is a disorder of glucose homeostasis characterized by impaired insulin action (insulin resistance) and impaired insulin production. It is widely held that genetic factors play a key role in the development of insulin resistance and NIDDM. Insulin resistance is inherited in as a non-Mendelian trait, the pathogenesis of which likely involves genetic heterogeneity and polygenic inheritance. We are interested in dissecting the genetic components of insulin resistance using mouse and human genetic models. This project focuses on the characterization of mice with targeted mutations in the pathway of insulin action. To generate a mouse model of insulin resistance, we have initially introduced a mutant allele of the insulin receptor in mice. Homozygosity for a null insulin receptor allele causes neonatal death from ketoacidosis, whereas heterozygosity for this mutation is associated with hyperinsulinemia, a sign of insulin resistance. These mice provide a valuable model to further characterize the mechanism of insulin action and its impairment in NIDDM. Three broad areas of investigation are being pursued: **a.** the generation of transgenic knock-outs in which expression of insulin receptors has been restored either in a tissue-specific fashion or with mutant insulin receptors; **b.** the derivation of cell lines from the knock-out mice to analyze the insulin signaling pathway, and **c.** the identifying of additional "diabetogenic" genes that interact with the insulin receptor gene in a polygenic model of insulin resistant-diabetes. Genetic abnormalities identified through these studies will be complemented by studies of NIDDM patients.

Project description:

Objective: a molecular dissection of the pathway of insulin action in transgenic mice to identify genes that play a role in the pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM).

Background:

Non-insulin-dependent diabetes mellitus (NIDDM) is currently viewed as a complex metabolic disorder with multiple causes. The metabolic hallmarks of NIDDM are peripheral insulin resistance and impaired insulin secretion. The two defects are intimately intertwined in most NIDDM patients. However, in longitudinal studies of patients at high risk of developing NIDDM, it appears that insulin resistance is the first identifiable metabolic abnormality. Insulin resistance is a genetically complex trait, i.e., it is not inherited in a Mendelian fashion. Thus, simple genetic models, such as studies of candidate genes, have failed to identify the gene(s) responsible for insulin resistance. Several aspects of insulin action appear to be impaired in NIDDM: in skeletal muscle, there is a defect in non-oxidative glucose disposal, as well as abnormalities of glucose uptake and phosphorylation. Likewise, there appear to be defects in insulin's ability to suppress free fatty acid release from the adipocyte, and glucose production in liver. The nature of the fundamental defect remains unclear. It is widely held that a defect at an early post-receptor step in insulin action could provide a unifying mechanism for insulin resistance. In recent years, the pace of discovery of new molecules and novel signaling mechanisms downstream of the IR has quickened considerably. However, studies of genes encoding proteins that participate in the insulin signaling systems have failed to implicate one or more of these genes as a major candidate for the ethiology of NIDDM. It is possible that, because of genetic heterogeneity, different genes are responsible for insulin resistance in different patients. Furthermore, the functional impairment caused by some genetic variations might be too subtle to be detected by the assays at our disposal, or may require the combined (epistatic) interactions of other mutant genes to give rise to the diabetic phenotype.

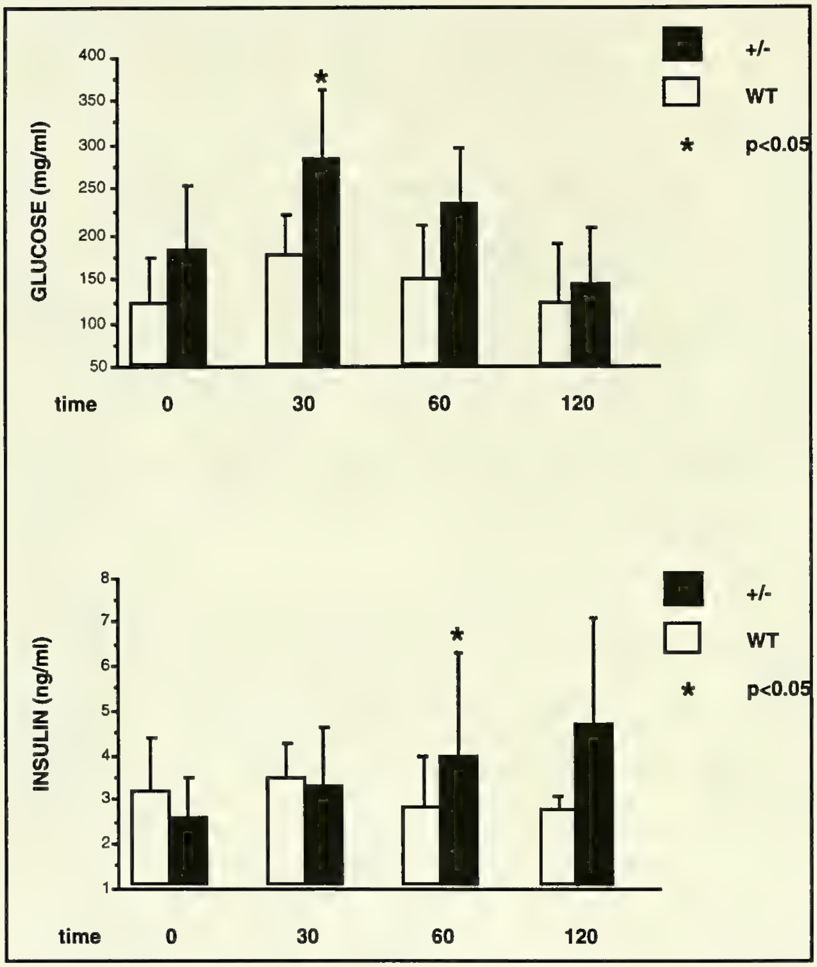
These hypotheses can be tested in vivo in mouse models. We have generated transgenic animal models of insulin resistance by way of targeted manipulations of genes important for insulin action. There are obvious advantages to mouse studies: embryo manipulation is both sophisticated and efficient; metabolic studies can be performed with great accuracy using glucose clamp techniques; and, through genetic crosses, it is possible to dissect the contribution of individual genes to the disease process. Finally, the establishment of a complete linkage map of the mouse genome has simplified the identification of new loci.

Major findings:

1. Insulin receptors are necessary for energy metabolism.

To generate a mouse model of insulin resistance, mice lacking functional insulin receptors have been derived in our laboratory using gene targeting in embryonic stem cells. Mice born without functional insulin receptors have normal features at birth; their intrauterine growth and development appear to be normal. However, upon feeding they develop dramatic metabolic alterations with hyperglycemia and hyperketonemia. Within few days, mutant mice die of diabetic ketoacidosis (DKA). Thus, lack of insulin receptors in mice impairs fuel metabolism. The early post-natal death caused by inactivation of the insulin receptor gene is reminiscent of other targeted inactivations of genes important for fuel metabolism, such as glucokinase and C/EBP-α.

The notion that mice can develop and be born without functional insulin receptors had been predicted, based on the description of four cases of leprechaunism, a genetic syndrome of extreme insulin resistance, in which children were born with homozygous non-sense mutations or deletions of the insulin receptor gene. Nevertheless, there are striking differences between insulin receptor-deficient mice and humans with similar mutations. The pathophysiologic basis of these differences remains speculative. In humans, fasting hypoglycemia and retarded growth are the hallmarks of severe insulin resistance. It is difficult to explain why mice present with hyperglycemia. A simple explanation is that newborn mice, unlike newborn humans, are constantly nursed by their mothers. Thus, there exists a constant flow of nutrients that prevent hypoglycemia, while exacerbating hyperglycemia and ketogenesis. To partially support this view, it should be noted that children with leprechaunism do experience transient hyperglycemia and ketosis following. In addition to the metabolic findings, leprechauns present with dysmorphic and virilized features that are absent in insulin receptor knock-out mice.



Next, we wanted to address whether heterozygosity for a mutant insulin receptor allele, with the associated decrease in insulin receptors number on the surface of target cells, gives rise to insulin resistance and diabetes. Interestingly, only **a subset of heterozygous IR mutant mice develops hyperinsulinemia.** We are studying the variability observed in the insulin-resistant phenotype by analyzing insulin receptor expression and insulin action in hyperinsulinemic IR heterozygous mice and comparing them with normoinsulinemic heterozygotes and wild-type controls (fig. 1).

In this experiment, we asked whether gestation, a condition known to be associated with insulin resistance and impaired insulin secretion, would further derange the metabolic response to a glucose challenge in IR heterozygous (+/-) mice. IPGTTs were performed in control (WT) and +/- mice at mid-gestation (p.c. day 15). As shown in the upper panel, glucose levels in +/- mice showed a trend toward higher values than in WT mice; however, the differences only reached a statistically significant value of $p<0.05$ at 30 min post-challenge. Likewise, insulin levels are consistently higher in +/- mice, but significantly so only at 60 post-challenge ($p<0.05$). Interestingly, insulin values in IR +/- mice show a delayed and sustained elevation at 60 and 120 min post-challenge, which could be interpreted to suggest that first phase insulin secretion in these animals is blunted. Thus, it appears that the genetic predisposition of IR +/- mice to develop diabetes may be unmasked by conditions of physiologically impaired insulin action. The large variance observed in insulin and glucose values in the two groups of mice suggests that further genetic analysis is needed to identify loci that contribute to the onset of insulin resistance in these mice.

Thus, it is important to test the hypothesis that the variations of insulin levels may be due to genetic effects of other loci acting in a synergistic fashion on insulin action.

2. A polygenic animal model of NIDDM

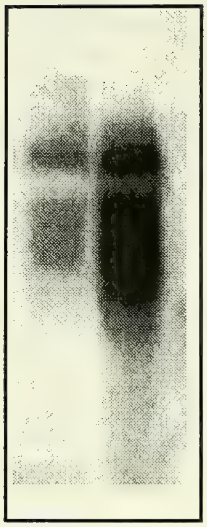
The hypothesis that insulin resistance may be polygenic in nature has been tested in preliminary experiments carried out in collaboration with Dr. C.R.Kahn's laboratory at the Joslin Diabetes Center. In these experiments, we have crossed IR heterozygous with mice heterozygous for an IRS-1 null allele. IRS-1 (insulin receptor substrate-1) is a protein substrate of the insulin and IGF-1 receptor kinases, and is thought to mediate insulin and IGF-1 signaling by way of interactions with adaptor molecules such as phosphatidylinositol (PI)-3-kinase, grb-2, sos, syp (tyrosine phosphatase 1B), and many others. Mice lacking IRS-1 are growth retarted and mildly insulin resistant, consistent with a model in which IRS-1 mediates bith the actions of insulin and IGF-1 receptors. Mice heterozygous for both an IR and an IRS-1 mutant allele develop all the hallmarks of insulin resistance: hyperinsulinemia and hyperplasia of pancreatic beta cells. Within two months of birth, they become frankly hyperglycemic, thus resembling very closely the pathogenesis of human diabetes. These findings are consistent with a polygenic mode of inheritance of NIDDM. This mouse model of NIDDM represents a significant advance over existing animal models of this condition, such as the ob/ob and db/db mice, which are primarily mouse models of obesity. A preliminary patent application for this “NIDDM mouse” has been submitted. In a broader context, lessons learned from this mouse model have a substantial impact on current gene mapping strategies in common diseases, because they demonstrate how two genes can interact epistatically in the pathogenesis of these disorders.

3. Insulin and IGF-1 actions in insulin receptor-deficient mice.

Mice lacking insulin receptors represent a valuable model to investigate pharmacological agents that can affect fuel metabolism in an insulin-independent fashion. Because of the growing interest in alternative treatments to insulin therapy for the cure and prevention of NIDDM, we have investigated several agents for their ability to improve glucose utilization in mice laking insulin receptors.

IGF-1 is a peptide of the insulin family that promotes cell growth and differentiation through its own receptor. IGF-1 has also been shown to exert insulin-like effects in vivo and in vitro. However, it is not clear whether the metabolic effects of IGF-1 are mediated by the IGF-1 receptor, or by the insulin receptor, to which IGF-1 binds with low affinity. Another possibility is that the metabolic actions of IGF-1 are carried out by hybrid insulin/IGF-1 receptors. To test these possibilities, IGF-1 was administered to newborn mice lacking insulin receptors, as well as heterozygous and wild-type controls. In all three treatment groups, IGF-1 lowered plasma glucose levels by ~50%. Despite the improved glycemic control, IGF-1 failed to improve the survival of insulin receptor-deficient mice. Thus, we have shown that IGF-1 can exert metabolic effects through its own receptor. We then investigated whether the metabolic effects of the IGF-1 receptor are mediated by the same signaling molecules utilized by the insulin receptor. Activation of PI-3-kinase is thought to play a pivotal role in insulin stimulation of glucose transport. We observed that in wild-type mice IGF-1 treatment increased the amount of PI-3-kinase that could be detected in anti-phosphotyrosine immunoprecipitates, suggesting that IGF-1 stimulates the association of PI-3-kinase with IRS-1, an important mediator of insulin and IGF-1 action. In contrast, IGF-1 failed to increase PI-3-kinase immunoprecipitation in mice lacking insulin receptors. Because ot the high doses of IGF-1 employed in our study, we believe that effect observed in wild-type mice can be explained by the activation of insulin receptors. The failure of IGF-1 to activate PI-3-kinase in insulin receptor-deficient mice represents an important preliminary observation. It suggest that elements downstream of the IRS-1 molecule may account for specificity of insulin vis-a-vis IGF-1 receptor signaling. This observation represents an important step to begin to dissect the molecular basis of signaling diversity betweeen these two highly homologus receptors. Further studies are in progress.

4. Role of insulin receptors in embryonic development.



Insulin receptors are expressed in the pre-implantation mouse embryo, and insulin has been shown to stimulate glucose utilization by isolated blastocysts. It is therefore somewhat surprising that embryonic growth and development are not affected by the lack of insulin receptors. Furthermore, it is known that lack of insulin receptors in humans is associated with severe growth retardation. The role of fetal insulin receptors is a matter of debate. One possibility is that insulin receptors regulate growth by regulating fuel metabolism. This possibility can be discounted in mice based on the phenotype of receptor-deficient animals, but has to be reckoned with in humans. In addition to the growth retardation observed in cases of extreme insulin resistance, it should be remembered that macrosomia is a well recognized complication of fetal hyperinsulinemia, a metabolic consequence of the diabetic pregnancy. An alternative explanation is that insulin receptors or hybrid insulin/IGF-1 receptors mediate some growth-promoting actions. However, the phenotype of nullizygous insulin receptor mice suggests that, during gestation, insulin receptors can be replaced by other receptors. Because of the close structural and functional homology between insulin and IGF-1 receptors, the IGF-1 receptor is a prime candidate to compensate for functions normally exerted by insulin receptors. Consistent with this hypothesis, we have observed that expression of the IGF-1 receptor gene is increased in mice embryos lacking insulin receptors (fig. 2, left lane represents wild-type mice, and right lane represents insulin receptor-deficient mice).

This question has been examined in more detail in collaboration with Dr. A. Efstratiadis's laboratory at Columbia University. Dr. Efstratiadis has shown that mice lacking IGF-1 receptors are severely growth retarded and die of unexplained complications shortly after birth. Likewise, mice lacking IGF-2 receptors die immediately after birth, but their body weight is increased by ~30%. Surprisingly, mice lacking both IGF-1 and IGF-2 receptors survive and have normal size. We have tested the hypothesis that the insulin receptor is the receptor responsible for survival of mice lacking both the IGF-1 and IGF-2 receptors. Mice lacking insulin as well as Igf-1 and Igf-2 receptors have been drived. Indeed, mice lacking both the insulin and the IGF-1 receptor are smaller than mice lacking either receptor alone (30% vs 45% for mice lacking IGF-1 receptors and 100% for mice lacking insulin receptors). Mice lacking all three receptors (insulin, IGF-1 and IGF-2) are even smaller than the double insulin/IGF-1 knock-out mice. These data, and the observation that IGF-1 receptor levels are increased in mice lacking insulin receptors, indicate that insulin receptors play an ancillary role in embryonic growth. It is likely that these actions of the insulin receptor are mediated in response to IGF-2 binding, since the knock-outs of the two ligands, insulin and IGF-1, suggest that the actions of these two hormones are only mediated by the respective receptors. It remains to be determined whether the growth-promoting actions of the insulin receptor are mediated by holodimeric insulin receptors (composed of two α- and two β-subunits) or by heterodimeric receptors composed by an insulin receptor α/ β monomer and an IGF-1 receptor α/ β monomer (see below, future course of studies).

5. Analysis of insulin action in cell lines derived from insulin receptor-deficient mice.

A molecular dissection of the mechanism of insulin action has been complicated by the lack of cell systems in which the broad array of biological responses to insulin can be assessed. Existing in vitro systems used to measure insulin actions on fuel metabolism are hardly amenable to genetic manipulations. On the other hand, systems prone to genetic manipulations do not possess the requisite intracellular machinery to mediate biological responses to insulin. For example, insulin's effects on fuel metabolism can be readily measured in cultured adipocytes and skeletal muscle cells. However, transfection studies in these cells are difficult to interpret, because of the presence of these large numbers of endogenous receptors and substrates. Cell lines with small numbers of either insulin receptors or IGF-1 receptors, on the other hand, generally lack other elements required to obtain a biological response such as insulin-dependent glucose uptake, and are, thus, hardly more informative.

We are actively engaged in the derivation of cell lines from insulin receptor-deficient mice, as a model to analyze the metabolic actins of insulin in vitro. We have succeeded at establishing permanent cell lines of SV40-transformed from these mice. These cells have been characterized for their ability to respond to insulin. The derivation of permanent lines of other insulin-responsive tissues has been more problematic. We have embarked on a collaboration with Dr. S. Sampson at Bar Ilan University (Israel). Dr. Sampson is very experienced in the establishment of primary cultures of skeletal muscle cells. In order to facilitate the establishment of a permanent cell line, we have introduced an SV40 T-antigen transgene into the insulin receptor deficient background by crossing the insulin receptor mutant mice with a transgenic mouse expressing the SV40 large T-antigen. Preliminary experiments are being performed.

Proposed course of the project:

1. Tissue-specific reconstitution of IR expression.

In most patients with NIDDM, insulin resistance is observed in skeletal muscle, liver, and adipose tissue. It is generally thought that insulin resistance in these tissues is central to the development of diabetes. It is less clear whether the primary event in the onset of insulin resistance is tissue-specific, or whether there is a generalized impairment of insulin action. In these experiments, we intend to dissect the role of different tissues in the pathogenesis of insulin resistance by generating mice in which IRs are selectively expressed in liver, fat, or skeletal muscle, but in no other tissue.

We will first address the issue of whether liver-specific expression of IRs is sufficient to rescue the lethal phenotype of mice homozygous for a null IR allele. Liver-specific expression will be obtained with an IR transgene driven by the rat albumin promoter/proximal enhancer. This reconstitution transgenic mouse will provide further insight into the pathophysiology of IR knock-out mice. In fact, it is not clear whether the ketotic hyperglycemia observed in nullizygous IR mice is primarily due to impaired glucose utilization in skeletal muscle and brown fat (there is little white fat at birth) or to increased hepatic glucose production. The liver plays a key metabolic role in the neonatal period. In rodents, insulin-dependent glucose utilization by skeletal muscle is established gradually during the first weeks of life, whereas the establishment of an efficient mechanism of hepatic glucose production is necessary to switch from fetal to post-natal nutrition. Thus, it is not entirely surprising that marked liver failure is observed in mice nullizygous for IR. We would predict that by restoring insulin action in the liver, it should be possible to prolong the survival of IR-deficient mice. Skeletal muscle, unlike liver, expresses IGF-1 receptors, so that glucose utilization might occur in muscle in an IGF-1-dependent manner.

It is important to determine whether normal insulin actions in the liver can protect against the development of NIDDM in mice heterozygous for the IR mutation. In humans with NIDDM, insulin fails to adequately suppress gluconeogenesis, thus contributing to the pathogenesis of fasting hyperglycemia. If transgenic mice with expression of IR restricted to the liver survive to adulthood, it should prove possible to ask whether insulin resistance in skeletal muscle and fat is sufficient to develop NIDDM .

The role of skeletal muscle and adipose tissue will be addressed by generating reconstitution transgenics in which IRs are expressed under the control of the GLUT 4 promoter, to obtain simultaneous expression in both tissues. This set of experiments is complementary to those described above with the liver-specific transgene. The obvious advantage of the GLUT-4 promoter is that two important insulin-sensitive tissues can be rescued at once. The use of the GLUT-4 promoter could be problematic, since the promoter is hormonally regulated. We have shown that the endogenous GLUT4 gene is expressed at normal levels in skeletal muscle and brown adipose tissue of IR-deficient mice. Thus, lack of IR should not prevent expression of the transgene. Furthermore, the main site of hormonal regulation of the GLUT-4 promoter is white adipose tissue, which is scarcely present at birth. The main GLUT-4-expressing tissues at birth are skeletal muscle and brown adipose tissue, which are less sensitive to hormonal regulation by insulin.

2. Site-directed mutants of the insulin receptor.

The IR has been extensively mutagenized in the last decade. However, the relevance of some mutations to insulin action in vivo is still unclear, largely because transfection experiments have been conducted in non-physiologic target cells for insulin. Expression of mutant IRs in transgenic mice will help define important domains for the biological actions of insulin in different tissues. This information could then be used to identify novel substrates, or tissue-specific differences in the pathway of insulin signaling. Two mutations will be studied initially using a eukaryotic promoter (GK) to drive ubiquitous expression of the transgenes (fig. 4):

a. Y960F. Tyr^{960} is a site of insulin-induced phosphorylation in the juxtamembrane domain of the IR. Mutations of this amino acid residue impair the ability of the mutant receptor to phosphorylate the signaling molecules IRS-1, IRS-2 and shc. These substrates play an important role in insulin signaling, even though the specific role of each one of them has not been elucidated yet. In vivo mutagenesis shows that mice lacking IRS-1 are growth retarded and hyperinsulinemic, suggesting that IRS-1 is important to mediate both the actions of insulin and IGFs. However, the presence of IRS-2 appears to compensate for lack of IRS-1. For example, insulin actions are severely blunted in skeletal muscle of IRS-1 deficient mice, but less so in liver, because of the presence of IRS-2 in that tissue at a higher level than in muscle. Thus, this reconstitution transgenic will enable us to address whether there exist tissue-specific differences in IR signaling.

b. The carboxy-terminal domain of the IR is the least conserved between insulin and IGF-1R. Thus, this is a potential site to regulate the diversity of responses to insulin and IGF1. Most site-directed mutagenesis studies have suggested that a deletion of this domain does not impair receptor autophosphorylation, kinase activity and biological responses to insulin. An interesting exception is that phosphorylation of the liver-specific substrate of the IR kinase pp120/ecto-ATPase requires the carboxy-terminal domain of the IR, and cannot be elicited by the corresponding domain of the IGF1 receptor. Furthermore, since studies demonstrating normal response to insulin in cells expressing this truncated mutant were conducted in non-physiological target tissues, their significance remains, in this investigator's opinion, controversial. We have developed and used a mutant receptor lacking the 43 carboxy-terminal amino acids. This construct will be engineered for expression in transgenic mice.

3. Interactions between IRs and other receptors of the same subfamily of tyrosine kinases.

This section dwells on the generation of multiple knock-outs of receptors of the IR family, and of a reconstitution transgenic to address the role of hybrid IR/ IGF-1R.

a. Reconstitution transgenic K1018M. The physiological role of hybrid receptors composed of an IR monomer and an IGF-1R monomer is incompletely understood. Transgenic mice experiments indicate that hybrid receptors composed of a normal and a mutant IR are assembled in vivo, whereas hybrid IR/IGF-1Rs have been detected at a much lower level. We have been unable to demonstrate hybrid receptors between normal and mutant insulin receptors. Therefore, we would like to address this question in a more physiological context. We plan to cross the IR null mice with transgenic mice expressing an ATP binding site mutant of the IR (K1018M). This mutation renders the IR totally inactive. Furthermore, it acts as a dominant negative mutation to inhibit function of a hybrid receptor composed by a normal insulin or IGF1 receptor and the mutant K1018M. Thus, if IR/IGF-1R hybrids form in vivo, it should be possible to detect their role in embryonic development.

Another question should be considered. In vitro experiments have shown that this mutation totally abolishes IR activity. Thus, we expect that this mutant will not be able to rescue the lethal phenotype. However, we should be open to the possibility that this prediction is not correct.

b. Crosses Between IR/Insulin Receptor-related Receptor (IRR) Knock-out Mice. The

IR subfamily of tyrosine kinases comprises an orphan member, the IR-related receptor (IRR). The function of this protein is unknown, as is the endogenous ligand. Dr. Luis Parada's laboratory has generated a knock-out mouse lacking IRR. This mouse is phenotypically normal. We have exchanged mice with Dr. Parada to cross the IRR knock-out with the IR knock-out. The rationale for this experiment is that the function of IRR may be exerted through hybrid receptors formed with either the insulin or the IGF1 receptor. Available evidence indicates that hybrid receptors form in cells expressing IRR and IR or IGF-1R.

Significance to Biomedical Research and the Program of the Institute:

In the US, NIDDM affects 16 million people, half of whom are undiagnosed. Total health care costs of this disease exceed $ 90 billion/ year. In addition to its long term complications, diabetes is a major cause of high risk pregnancy, complicating up to 5% of all pregnancies in the US. Furthermore, maternal diabetes is a major risk factor for the development of childhood obesity and NIDDM in adult life. There is growing consensus that NIDDM should be prevented by combining a pharmacological approach with life style modifications. Because of the genetic predisposition to developing NIDDM, the identification of individuals at risk is an important part of the prevention effort. The identification of mechanisms of insulin resistance and genes involved in its pathogenesis is central to our developing a coherent prevention strategy in individuals at risk of NIDDM.

Bibliography

Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI and Westphal H. Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. Nat Genet 1996;12:106-9

Accili D. Insulin receptor knock-out mice. Trends Endocrinol. Metab. in press.

Bruening J, Winnay J, Bonner-Weir S, Accili D and Kahn CR. Creating polygenic models of insulin resistance and NIDDM. J. Invest. Med. 1996;44(3):258A

Di Cola G, Cool MH and Accili D. Normal metabolic responses to IGF-1 in mice lacking insulin receptors. Annual Meeting of the Endocrine Society 2:749: abstract OR66-1

Patent Application:

Kahn CR, Bruening J, Accili D. U.S. Serial 60/019,412. A novel mouse model for non-insulin dependent (Type II) diabetes mellitus. Provisional patent.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00640-01 DEB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Developmental Endocrinology of Bone and Mineral Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Baron, M.D.	Senior Clinical Investigator	UGD, SDE, DEB, NICHD
Others:	F. DeLuca, M.D.	IRTA Fellow	UGD, SDE, DEB, NICHD
	E. Mancilla, M.D.	Visiting Fellow	UGD, SDE, DEB, NICHD

COOPERATING UNITS *(if any)*

Metabolic Disease Branch, NIDDK (A. Spiegel), Medical Genetics Branch, NCHGR (C. Francomano); Mayo Clinic, Rochester, MN (D. Zimmerman); NPS Pharmaceuticals, Salt Lake City, UT (F. Fuller)

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Developmental Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to understand the cellular and molecular mechanisms governing bone growth and mineral metabolism. To understand the molecular and cellular mechanisms governing longitudinal bone growth, the investigators developed several experimental approaches including an in vivo experimental system that allows infusion directly into the growth plate, methods for extracting and analyzing specific mRNA transcripts from growth plate cartilage, and a fetal bone organ culture system. Using these system, the investigators demonstrated that glucocorticoid inhibits longitudinal bone growth by a direct action on the growth plate, and found unexpectedly that glucocorticoid increases growth hormone receptor mRNA in growth plate and liver. The investigators further demonstrated that catch-up growth, which was previously thought to be regulated by a neuroendocrine mechanism, actually results from a mechanism intrinsic to the growth plate. They have provided evidence suggesting that fibroblast growth factors attract vascular and bone cell invasion from the adjacent metaphyseal bone, processes critical for endochondral bone formation.

In the area of mineral metabolism, this research group recently demonstrated that activating mutations in the Ca^{2+}-sensing receptor gene cause autosomal dominant and sporadic hypoparathyroidism. Because the Ca^{2+}-sensing receptor is expressed not only in parathyroid cells but also in kidney cells, where it negatively regulates calcium reabsorption, the investigators predicted and experimentally confirmed that patients with these mutations have hypercalciuria even at low serum calcium concentrations. Their findings have important implications for the genetic counseling and choice of therapy for these patients. Current experiments are designed to identify additional mutations, to elucidate the molecular mechanism of activation by expressing the mutated receptors in cell culture, and to further explore the physiological and clinical consequences of these mutations.

Project Description:

Objectives:

The goal of this project is to understand the cellular and molecular mechanisms governing bone growth and mineral metabolism, with a particular focus on the molecular signals regulating longitudinal bone growth. This knowledge will be applied to improve medical treatment in disorders of bone growth and in disorders of mineral metabolism. Through the study of longitudinal bone growth, the investigators seek to uncover general principles of developmental biology since the cellular processes underlying bone growth, such as cell proliferation, terminal differentiation, angiogenesis, and cell migration, are essential for development in other tissues.

Methods Employed:

1. Quantitation of Longitudinal Bone Growth

This group developed a new radiologic method to quantitate longitudinal bone growth in vivo. Metal pins were surgically implanted in the proximal tibia of 5-6 week old male New Zealand White rabbits, just proximal and just distal to the growth plate. Serial radiographs were obtained on the day of surgery and on subsequent days. Each radiograph was examined under a dissecting microscope, and the distance between pins was measured using an eyepiece micrometer. The distance between pins was used to calculate growth rate. This method proved highly precise with a measurement error of 0.02 mm.

2. In vivo Growth Plate Infusion

The investigators also developed a method to infuse substances directly into the rabbit proximal tibial growth plate.They surgically inserted a 27-gauge needle into the proximal tibial growth plate near the junction with the bony epiphysis. The blunt end of the needle was attached to a catheter which was connected to an osmotic pump. For some experiments, infusion sets were placed for both tibiae. One side received the agent of interest while the other side received vehicle as a control.

3. Isolation of RNA from Growth Plate Cartilage and Quantitation of Specific mRNA Levels

Because no suitable procedure existed, the investigators developed a new method to isolate RNA from the growth plate. The tissue was obtained from the proximal tibiae and distal femurs of 5-week old male rabbits. The bones were fractured along the growth plate and submerged in a guanidinium thiocyanate solution to inhibit RNase activity. The growth plate cartilage was then scraped from both the metaphyseal and epiphyseal surfaces. Tissue was homogenized in guanadinium thiocyanate solution, digested with proteinase K to degrade proteoglycans, and subjected to organic extraction, ethanol precipitation, and reprecipitation with 4 M LiCl. This rapid, simple method provided a high yield of intact RNA with good purity. Reasonable yields of RNA (approximately 0.5 mg per mg tissue) were obtained even from 25 mg of tissue, which represents approximately half of a growth plate. Specific RNA transcripts were quantitated by a solution hybridization assay.

4. Long Bone Organ Culture

Tibiae or metatarsals were isolated from rats on embryonic day 21 and cultured intact in minimum essential medium enriched with either fetal calf serum or glutamine, ascorbic acid, and albumin at 37 C for up to 5 days. Growth was quantitated using a dissecting microscope and micrometer. Standard histologic methods were used.

5. Analysis of Human Genomic DNA

To identify mutations in human genomic DNA, standard methods were used, including polymerase chain reaction amplification, heteroduplex analysis using partially denaturing polyacrylamide gels, dideoxy sequencing, and restriction analysis.

6. Expression of Mutant Receptors in Cultured Cells

Mutations were created in the Ca^{2+}-sensing receptor cDNA by site-directed mutagenesis. Expression plasmids containing the cDNA's were transiently transfected into HEK 293 cells. Cells were loaded with fura-2 and assayed for their ability to increase cytoplasmic Ca^{2+} concentrations in response to extracellular Ca^{2+}.

Progress:

1. Saltatory Versus Continuous Growth

Growth has generally been assumed to be a continuous process, the cumulative sum of millions of unsynchronized cell replications. However, a recent report in *Science* challenged this view, concluding instead that human linear growth occurs in sudden spurts, separated by long periods with no measurable growth.

The validity of the saltatory growth model is still open to question because of limitations in the initial study. To address these limitations, the investigators first used the rabbit growth model that they had developed. It allowed much greater accuracy than could be achieved in the human, and lent itself to a blinded experimental design. The frequency distribution of observed daily growth velocities was inconsistent with the saltatory growth hypothesis and was instead consistent with continuous growth.

To test the saltatory growth hypothesis in humans, the investigators devised a photographic method to measure lower leg growth in infants, analogous to the radiographic method used in rabbits. Using this new method as well as standard methods, they measured daily growth in five healthy infants over a period of one month. For all four types of measurements (crown-heel length, knee-heel length, head circumference, and weight) the daily growth velocity frequency distributions approximated a single Gaussian distribution consistent with continuous growth. All of the distributions were significantly different from the predictions of the saltatory model. The data were also analyzed using three other mathematical methods involving skewness of the distributions, correlation between individual growth measures, and linear spline modeling. By all four statistical approaches, the data were incompatible with the saltatory model, suggesting instead that human growth occurs continuously.

2. Regulation of Growth Rate by Glucocorticoid

Excess glucocorticoid inhibits longitudinal bone growth. Proposed explanations for this growth inhibition include both direct local effects of glucocorticoid on the growth plate and indirect systemic effects. To address this controversy, the investigators administered dexamethasone for 7 days directly into the growth plate of 5-week old rabbits. In each animal, one proximal tibial growth plate received dexamethasone phosphate and the contralateral growth plate received vehicle. Growth velocity was measured radiographically as described above. Local dexamethasone administration decreased proximal tibial growth rate by 77% compared with the contralateral vehicle-treated tibia ($p<0.0001$). These data indicate that excess glucocorticoid causes a rapid potent inhibition of growth by a direct local action on the growth plate.

One way that glucocorticoid might act locally to suppress growth is by rendering target tissues insensitive to GH. The investigators hypothesized that this glucocorticoid-induced GH insensitivity is due to decreased GH receptor mRNA levels. To test this hypothesis, they treated 4.5-week old male rabbits with ip dexamethasone or vehicle and measured GH receptor mRNA levels (by RNase protection assay). Contrary to their hypothesis, dexamethasone, administered in growth-suppressing doses, did not decrease GH receptor mRNA levels in liver or growth plate. Instead, a tissue-specific stimulation of GH receptor mRNA levels was observed in growth plate and liver, target tissues important for linear growth, but not in kidney. These data suggest that glucocorticoid-induced growth suppression cannot be explained by decreased GH receptor mRNA levels.

3. Catch-up Growth

The prevailing explanation for catch-up growth involves a central nervous system mechanism that compares actual body size to an age-appropriate set point and adjusts growth rate accordingly. In contrast, the investigators hypothesized that the mechanism governing catch-up growth resides not in the central nervous system but rather in the growth plate. To test this hypothesis, they asked whether transient suppression of growth by excess glucocorticoid within a single growth plate would lead to local catch-up growth. They administered dexamethasone directly into the proximal tibial growth plate of 6-week old rabbits and vehicle into the contralateral growth plate. Dexamethasone slowed the proximal tibial growth rate during the 4-week infusion compared to the contralateral vehicle-treated control. After the infusion ended, the growth rate of the dexamethasone-treated side not only normalized but actually surpassed that of the control side, thus correcting approximately half of the growth deficit. Since the catch-up growth was unilateral, it could not be explained by the prevailing neuroendocrine hypothesis. A neuroendocrine mechanism, or any systemic mechanism that involved circulating factors, would have affected all growth plates and thus could not by itself account for the observed anatomic specificity. The data instead suggest that the capacity for catch-up growth is intrinsic to the growth plate.

4. Role of Fibroblast Growth Factors in Longitudinal Bone Growth

Fibroblast growth factors (FGF's) and FGF receptors are expressed by growth plate chondrocytes. Addition of FGF's to growth plate chondrocyte culture stimulates the anabolic processes underlying chondrogenesis, including synthesis of DNA, total protein, collagen, and glycosaminoglycan and inhibits terminal (hypertrophic) differentiation. In humans, mutations in FGF receptors cause skeletal dysplasias, thus confirming the importance of these growth factors in bone growth.

To examine the effects of FGF's in vivo, the investigators infused FGF-2 directly into the rabbit proximal tibial growth plate. The contralateral proximal tibial growth plate received an infusion of vehicle as a control. The FGF infusion accelerated local vascular invasion. Ossification of growth plate cartilage was also accelerated, advancing precisely in step with the increased vascular invasion. These results suggest the possibility that FGF endogenous to the growth plate attracts the normal invasion of vessels and bone cells from adjacent metaphyseal bone.

5. Ca^{2+}-Sensing Receptor Mutations

This research group identified Ca^{2+}-sensing receptor (CaR) gene mutations in four families with autosomal dominant hypoparathyroidism. All were heterozygous missense mutations. Because the CaR inhibits parathyroid hormone secretion, these mutations must activate the CaR. The resulting phenotype varies from asymptomatic to life-threatening, depending on the specific mutation.

The investigators also demonstrated that de novo mutations in this gene cause sporadic hypoparathyroidism. To date they have identified de novo mutations in three of the six patients with sporadic hypoparathyroidism followed in the NICHD endocrinology clinic. Identification of these mutations in sporadic cases defines the risk for the patient's future offspring, thus allowing appropriate genetic counseling.

Since the CaR also inhibits calcium reabsorption from the glomerular filtrate, the investigators predicted that activating CaR mutations would cause hypercalciuria even at low serum calcium concentrations. They empirically confirmed this prediction by comparing calcium excretion in patients with activating mutations to patients with other forms of hypoparathyroidism. This finding has an important clinical implication; patients with activating CaR mutations are at greater risk for hypercalciuria and its adverse consequences than are other hypoparathyroid patients, and thus they may benefit from treatments that lower urinary calcium excretion.

Significance to Biomedical Research and the Program of the Institute:

Understanding the molecular and cellular mechanisms that govern normal longitudinal bone growth may provide insight into the many disorders of bone growth including skeletal dysplasias and idiopathic growth failure. For example, the role of fibroblast growth factors in longitudinal bone growth was, until recently, only studied in cell culture and animal models. It is now clear that FGF action is critically important in human bone growth and that defects in this system lead to multiple skeletal dysplasias.

In addition, the cellular processes underlying longitudinal bone growth, such as cell proliferation, terminal differentiation, angiogenesis, and cell migration, are essential for embryonic development in other tissues. By exploring these mechanisms in bone, the investigators therefore hope to uncover general principles of developmental biology. These fundamental developmental principles, in turn, may provide insight into a wide range of human disorders, including developmental anomalies and neoplasia in non-skeletal tissues.

Similarly, understanding the molecular mechanisms governing calcium, magnesium, and phosphorus physiology may lead to improved clinical care for patients with disorders of mineral metabolism. In fact, this group's studies of the Ca^{2+}-sensing receptor have already led to the identification of a new subtype of hypoparathyroidism with implications for genetic counseling, prognosis, and choice of therapy.

Proposed Course:

1. Role of Fibroblast Growth Factors in Longitudinal Bone Growth

An activating mutation of the type 3 FGF receptor (FGFR3) impairs longitudinal bone growth in patients with achondroplasia. However, addition of FGF to cultured growth plate chondocytes stimulates chondrocyte proliferation. The investigators hypothesize that this discrepancy occurs because the cultured cells lack the spatial organization and intercellular interaction present in the intact growth plate. To test this hypothesis, they have turned to a long bone organ culture model. They will treat these explants with FGF and measure the resulting longitudinal bone growth, cell proliferation, and matrix production.

In collaboration with Dr. Clair Francomano in the Human Genome Project, the investigators are testing the hypothesis that mutations in FGFR3 can cause not only thanatophoric dysplasia, achondroplasia, and hypochondroplasia as previously described, but also extreme short stature without obvious skeletal deformity. Thus, they are screening children with extreme short stature for these mutations. The incidence of specific mutations in this population will be compared to the incidence in the normal population. Also family studies will be performed to determine whether any sequence alterations cosegregate with short stature.

2. Spatial Orientation and Polarity of the Growth Plate

When proliferative zone chondrocytes divide, daughter cells separate in a direction parallel to the long axis of the bone. As a result, cell clones are arranged in columns parallel to this axis. The signals that allows the cells to distinguish the long axis from other directions is not known. The investigators hypothesize that growth plate orientation and polarity are determined by a chemical gradient across the growth plate. Such a chemical gradient would arise if cells near the metaphyseal border elaborate a diffusible factor which is removed or degraded in the growth plate or in the adjacent epiphysis. Alternatively, this putative growth plate orienting factor (GPOF) could be elaborated by cells near the epiphyseal border and removed or degraded in the growth plate or metaphysis.

A recent report in a Chinese journal indicates that growth plate chondocytes which are dispersed and physically reaggregated will spontaneous reorganize into a structure resembling a normal growth plate. The investigators will attempt to replicate this finding. If true, it provides great insight into the mechanisms governing spatial orientation, and the system can be used as a model to delineate the underlying molecular and cellular interactions.

If this method cannot be replicated, growth plate autotransplantation will be used to investigate these questions. In young rabbits, the distal ulnar growth plate can be successfully isolated and reimplanted in the original bed. The investigators will excise the distal ulnar growth plate, invert it, and reimplant it in the original bed. If the GPOF originates within the growth plate, the growth plate will maintain its inverted histologic architecture (the hypertrophic chondrocytes will be found near the epiphyseal border) and the epiphysis rather than the metaphysis will increase in length. If, on the other hand, the GPOF originates in the adjacent bone, then the growth plate will reorient itself and normal metaphyseal elongation will result. With these and other analogous experiments, the investigators hope to identify the anatomic source and chemical nature of the putative GPOF.

3. Apoptosis in Growth Plate

The fate of the terminal hypertrophic chondrocyte has been debated for more than 40 years. It is not clear whether this chondrocyte which is adjacent to the chondro-osseous junction converts to an osteoblastic phenotype, thus contributing to bone formation or undergoes cell death. Recent electron and light microscopic studies suggest that the terminal hypertrophic chondrocyte may undergo apoptosis. To test that hypothesis, apoptotic cells will be labeled in situ using terminal deoxynucleotidyl transferase. Using this method the investigators will look for evidence of apoptosis in proximal tibiae from 4-week old Sprague-Dawley rats.

If the terminal hypertrophic chondrocyte does, in fact, undergo apoptosis, the investigators will attempt to determine the signal that initiates this programmed cell death. They hypothesize that retinoic acid (RA) produced in the metaphysis induces apoptosis of the hypertrophic chondrocytes as they reach the metaphyseal border. This hypothesis would explain why administration of RA in vivo causes rapid disappearance of the growth plate. It would also be consistent with the observation that RA induces apoptosis in many cell types including the limb bud mesenchymal cell, a chondrocyte precursor. To test the hypothesis, they will treat 4-week old rats with all-trans RA and then perform the in situ assay for apoptosis. Analogous studies will be performed in long bone organ culture.

4. Molecular and Clinical Characterization of Additional Hypoparathyroid Patients

To define further the spectrum of phenotypes and to perform genotype-phenotype correlations, the investigators have studied additional patients with hypoparathyroidism. They now have identified mutations in a total of four families with autosomal dominant disease and three patients with sporadic disease. They are currently completing the genetic and clinical characterization of these patients and their families.

5. Ca^{2+}-Sensing Receptor Mutations: Expression Studies

To explain the phenotype, Ca^{2+}-sensing receptor (CaR) gene mutations associated with hypoparathyroidism must activate the receptor. Ca^{2+} is thought to bind to extracellular portions of the CaR, and thus the investigators hypothesized that extracellular mutations activate the receptor by increasing its affinity for Ca^{2+}. In contrast, G-proteins are thought to interact with transmembrane and intracellular portions of the receptor, and thus they hypothesized that mutations in these regions would activate the receptor by allowing it to interact with a G-protein even in the absence of Ca^{2+}, that is, constitutively activate it.

To test these hypotheses, the investigators are using site-directed mutagenesis to introduce these mutations in the CaR cDNA, and then expressing these constructs in HEK 293 cells. They then vary the extracellular concentration of calcium and quantitate the resulting receptor activation by measuring cytoplasmic calcium levels. Preliminary results indicate that two extracellular mutations cause a left-shift in the dose-response curve. This observation supports their hypothesis that extracellular mutations increase the affinity of the receptor for calcium. The investigators are currently studying the molecular effects of the other mutations.

Protocols:

Animal

96-012	De Luca	Role of fibroblast growth factor, transforming growth factor β, and insulin-like growth factor in growth plate cartilage.
94-006	Yanovski	Effects of retinoic acid on rat epiphyseal growth plate

Human

94-CH-0102 Baron Children with endocrine-related conditions

This protocol allows non-invasive study of children with a wide variety of disorders. It provides for an assessment of their altered endocrine physiology and for molecular genetic studies. It also serves as a vehicle to evaluate patients as part of the NICHD pediatric endocrinology training program.

Publications:

Baron J. Longitudinal bone growth: experimental methods. In: Thomas JA, ed. Endocrine Methods. Academic Press Inc, San Diego, 1996;81-8.

Baron J, Winer KK, Yanovski JA, Cunningham AW, Laue L, Zimmerman D, Cutler GB Jr. Mutations of the Ca^{2+}-sensing receptor gene cause autosomal dominant and sporadic hypoparathyroidism. Hum Mol Genet 1996;5:601-6.

Heinrichs C, Munson PJ, Counts DR, Baron J. Patterns of human growth. Science 1995;268:442-4.

Klein KO, Demers LM, Santner SJ, Baron J, Cutler GB Jr, Santen RJ. Use of ultra-sensitive recombinant cell bioassay to measure estrogen levels in women with breast cancer receiving the aromatase inhibitor, letrozole. J Clin Endocrinol Metab 1995;80:2658-60.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00641-01 DEB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Physiology and Genetics of Obesity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	J.A. Yanovski	Sr. Clinical Investigator	UGO, SWH, DEB, NICHD
Others:	K. Rother	Clinical Associate	OTC, DEB, NICHD

COOPERATING UNITS *(if any)*

S. Yanovski, Program Director, DEM, NIDDK; A. Boyle, Special Volunteer, DEM, NIDDK; M. Flood, Nutritionist, NUTR, CC; D. Ford, Nutritionist, NUTR, CC; J. Reynolds, Chief, Clinical Studies, NM, CC; N. Avila, Radiologist, DR, CC

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Womens Health, Unit on Growth and Obesity

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Substantial evidence suggests that risk factors and therapeutic approaches derived from the study of Caucasians may not be accurate or effective when applied to individuals of differing ethnicity or race. Many Minority populations in the US, including African American women, have a markedly increased risk from obesity, diabetes mellitus, hypertension, cardiovascular disease, and mortality. This unit investigates the genetic, physiologic, metabolic, and behavioral factors involved in determining weight gain in African Americans and Caucasians. We have found that ACTH levels of African American women, men, and children following ovine CRH are two-fold higher than those of Caucasians, despite no differences in cortisol secretion, that visceral adipose tissue burden in obese African American women is significantly smaller than that of obese Caucasian women, and that less total abdominal, subcutaneous abdominal, and visceral adipose tissue in non-obese African American prepubertal girls than in Caucasian girls, findings that imply the relationship between visceral fat and the complications of obesity are different in African Americans and Caucasians. The susceptibility to weight gain in African American may also result from differences in metabolic efficiency: we have also found that resting energy expenditure is approximately 90 kcal/d less in African American than in Caucasian normal weight girls.

Related to the significantly increased prevalence of non-insulin-dependent diabetes mellitus in African Americans compared with Caucasians, this unit has investigated differences in insulin sensitivity, glucose disposal, and fat cell metabolism. In normal weight children we have found a correlation of the subcutaneous adipose tissue depot with insulin levels before and after an oral glucose tolerance test only in African Americans. It is unknown whether these differences are found in obese children or in children at high risk for developing obesity.

We will compare body composition, energy expenditure, insulin sensitivity and glucose disposal of obese African American and Caucasian prepubertal obese children and non-obese children of obese African American and Caucasian parents, in order to characterize the timing and nature of factors that may contribute to the prevalence of obesity and its complications. We will also relate serum levels of the body-fat related protein, leptin, to these measures, and characterize the role of the beta-3 adrenergic receptor and leptin signalling pathway in these children's obesity. We will then attempt to follow the growth of these children for the next 15 years. These studies will aid in the rational development of treatments for, and programs for the prevention of, obesity in minority populations.

Project Description:

Objectives:

1. To define and quantify the genetic, physiologic, metabolic, and behavioral characteristics leading to obesity in major US populations.

2. To devise sub-population-specific risk predictors to enable identification of children and adults at risk for the development of obesity and its complications.

3. To use this information to develop rational interventions specifically targeted to treat and prevent obesity in varied US populations.

Introduction

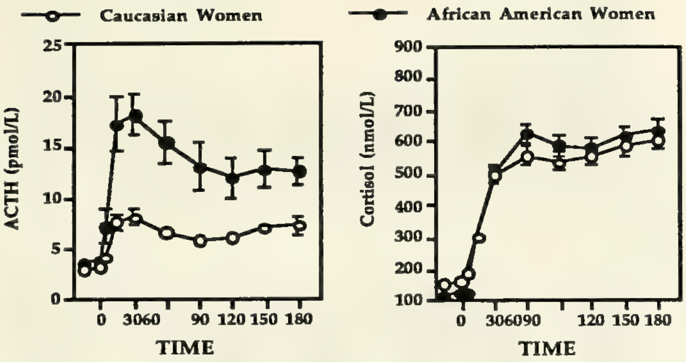
Many Minority populations in the US have a markedly increased risk for obesity and its associated disorders, such as diabetes mellitus, hypertension, and atherosclerotic cardiovascular disease. Obesity-related non-insulin-dependent diabetes mellitus and its complications, including hypertension, nephropathy, retinopathy, peripheral vascular disease, and death, are increased as much as 6-fold in minority populations such as African Americans. Thus the prevention and treatment of obesity in minority populations is a national problem of the utmost importance. Much data suggests that physiologic differences may contribute both to the greater prevalence and more refractory nature of obesity in the African American population. We have investigated the physiology, body composition, metabolism, and energy expenditure of African Americans and Caucasians, in an effort to elucidate the similarities and differences in the mechanisms underlying the onset of obesity and its associated comorbidities, to allow development of rational interventions specifically targeted to prevent and treat obesity.

Major Findings:

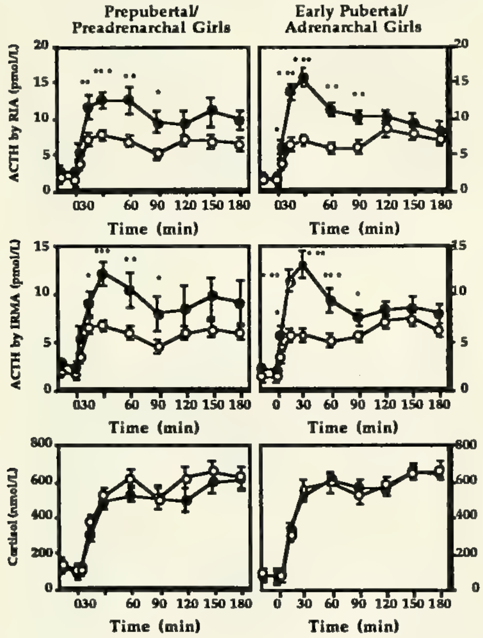
1. Physiology.

Alterations in the activity of the hypothalamic-pituitary-adrenal (HPA) axis have been associated with obesity in humans and animals. To explore possible mechanisms responsible for the higher prevalence of obesity and its associated comorbid conditions in the black population, we studied the HPA axis of 18 black and 30 white weight- and age-matched nonobese and obese women. Waist-to-hip ratio, 24-hour urine free cortisol excretion, plasma cortisol responses to dexamethasone, and plasma adrenocorticotropin (ACTH) and cortisol responses to 1 µg/kg ovine corticotropin-releasing hormone (CRH) were determined. There were no racial differences in waist-to-hip ratio, 24 h urine free cortisol excretion, dexamethasone suppressibility of plasma cortisol, baseline plasma cortisol and ACTH concentrations, or plasma cortisol response to CRH. However, CRH-stimulated plasma ACTH concentrations, measured in an extraction polyclonal radioimmunoassay, were significantly greater in blacks than in whites at all time points, beginning 5 minutes after administration of CRH (area under curve [AUC] 2463 ± 288 pmol/L • min in blacks vs 1185 ± 78 in whites, $p<0.001$). These differences persisted when ACTH was measured by a 2-site direct IRMA measuring the intact $ACTH_{1-39}$ molecule (AUC 1292 ± 177 pmol/L • min in blacks vs 504 ± 95 in whites, $p<0.002$). There was no significant correlation between body mass index and either cortisol or ACTH AUCs for either race, with blacks showing persistently elevated AUC for ACTH as compared to whites, regardless of weight. We concluded that there are differences in the HPA axis of black and white women that might be of importance in understanding differences in the prevalence of obesity in African Americans and Caucasians.

Similar findings were obtained in a study comparing African American and Caucasian mend. CRH-stimulated plasma ACTH concentrations, were significantly greater in African Americans at all time points between 30 and 180 minutes after administration of CRH (area under curve [AUC] 1796 ± 245 pmol/L • min in Blacks vs 1278 ± 121 pmol/L • min in Whites, $p<0.001$).



To determine whether these differences in ACTH secretion are also present in prepubertal and early pubertal girls, we studied the HPA axis of 19 African American and 19 Caucasian normal weight girls (age 7-10y) who were matched for body mass index, age, and socioeconomic status. There were no racial differences in waist circumference, waist-to-hip ratio, fasting glucose or insulin, baseline free or total plasma cortisol levels, baseline ACTH concentrations, or the plasma cortisol response to CRH. However, CRH-stimulated plasma ACTH, measured in a polyclonal radioimmunoassay, was significantly greater in prepubertal and early pubertal African American girls than in Caucasian girls at all time points between 15 and 90 minutes after administration of CRH (area under curve [AUC] 1754 ± 121 pmol/L • min in African Americans vs 1304 ± 124 pmol/L • min in Caucasians, p<0.001). This difference was confirmed using an immunoradiometric assay believed to be specific for intact ACTH (AUC 1634 ± 139 pmol/L • min in African Americans vs 1224 ± 104 pmol/L • min in Caucasians, p<0.001). We concluded that there are differences in the HPA axis of prepubertal and early pubertal African American and Caucasian girls similar to those found previously in adult women. As the timing of most weight gain in African American females is related to the onset of puberty, the significance of these differences in ACTH secretion for understanding the weight gain seen during adolescence in African Americans remains unclear.



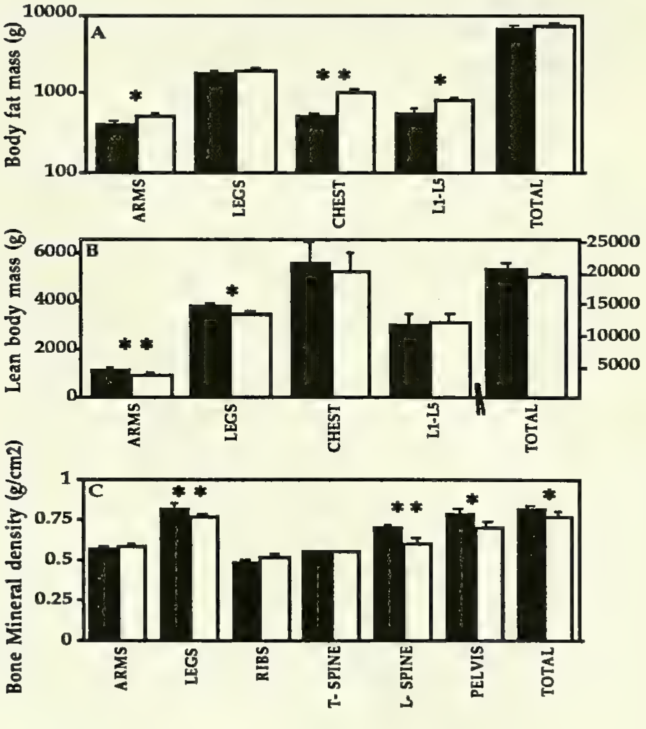
Plasma ACTH and Cortisol response to CRH (1 µg/kg) in Caucasians (o) and African Americans (•) in girls without evidence of either puberty or adrenarche, and in those with early signs of puberty. * p<0.05, ** p<0.01, *** p<0.001.

2. Body Composition.

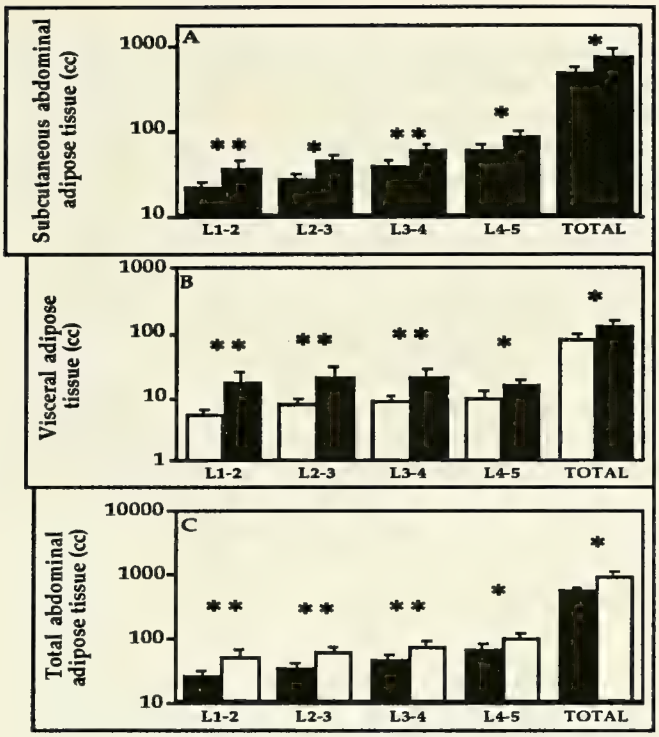
We have previously shown significant differences in the degree and distribution of visceral fat in African American and Caucasian women, as measured by CT scanning. We found less visceral adipose tissue in weight-matched obese African American women, compared to Caucasian women. Obese African American women, with similar total body fat and waist circumference to Caucasian women, had 25% less visceral adipose tissue before, during, and after weight loss.

To examine whether these racial differences in fat distribution are dependent on the hormonal changes that result from puberty, we studied African American and Caucasian girls, using dual energy x-ray absorptiometry and abdominal magnetic resonance imaging to determine whether there are racial differences in body composition before the onset of puberty.

20 African American and 20 Caucasian normal-weight girls ages 7-10, were matched for weight, body mass index (BMI), bone age, chronological age, breast Tanner stage, and socioeconomic status. Each underwent anthropometric measurements, bioelectrical impedance analysis, dual energy x-ray absorptiometry (DXA), and abdominal magnetic resonance imaging (MRI) for determination of total (TAT), visceral (VAT), and subcutaneous (SAT) adipose tissue. There were no differences between groups in absolute waist circumference or waist-to-hip ratio, but waist-to-thigh ratio was smaller in African American as compared to Caucasian girls. African American girls had greater bone mineral density and less TAT, VAT and SAT than Caucasians. We concluded that there are significant racial differences in body composition in African American and Caucasian prepubertal girls.



Body fat mass (A), lean body mass (B), and bone mineral density (C) determined by DXA in Caucasians (open bars) and African Americans (closed bars). * p<0.05, ** p<0.005.



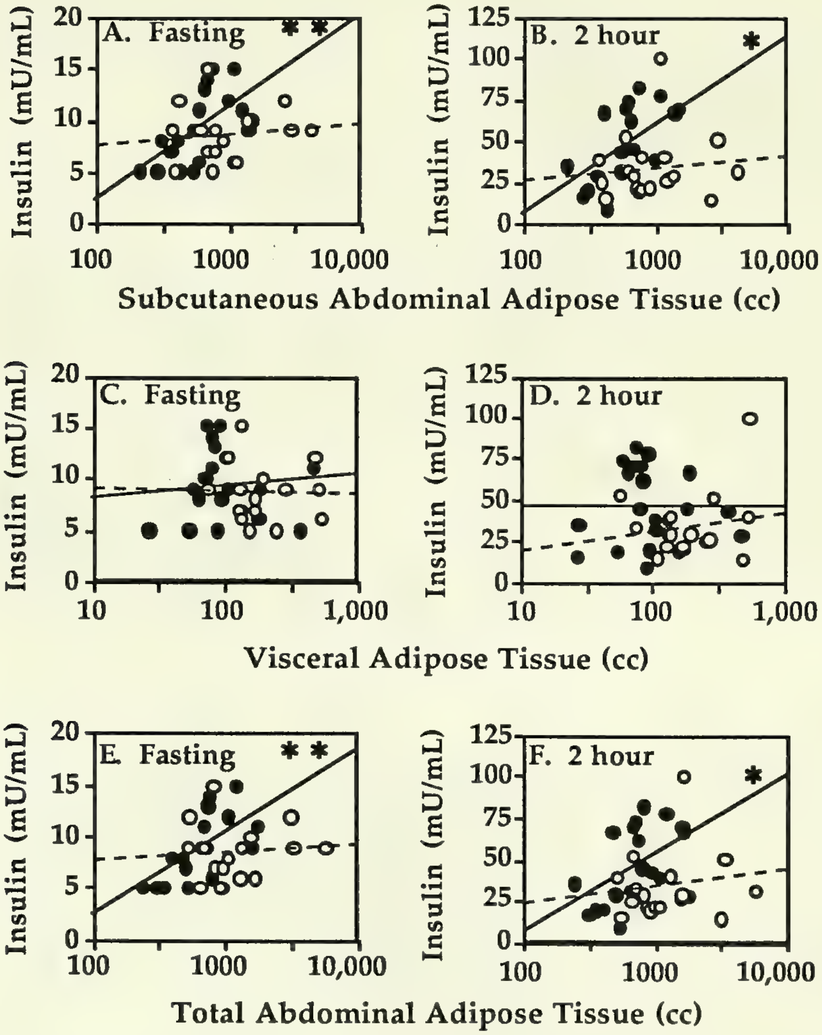
Subcutaneous abdominal (A), visceral (B), and total abdominal (C) adipose tissue determined by MRI in Caucasians (open bars) and African Americans (closed bars). * $p<0.05$, ** $p<0.005$

The results of this study showed that, at similar body weights, prepubertal African American girls have less adipose tissue than Caucasian girls in several regions of the body. Although this study examined children within the 15th to 85th percentiles for body mass index, we hypothesize that these results will also hold true for children of greater body mass index. Should studies of African American and Caucasian children of greater body mass index also find greater lean body mass and lower body fat mass in African Americans, such results may indicate that definitions of obesity based primarily on the relationship of weight with adiposity in Caucasian populations may not adequately reflect the body composition of children in other racial or ethnic populations. The current study is complementary to studies in adults that have found significant differences in body composition between Caucasians and African Americans, Asian Americans, and Hispanics, and are consistent with the hypothesis that there are genetic bases for racial differences in body composition.

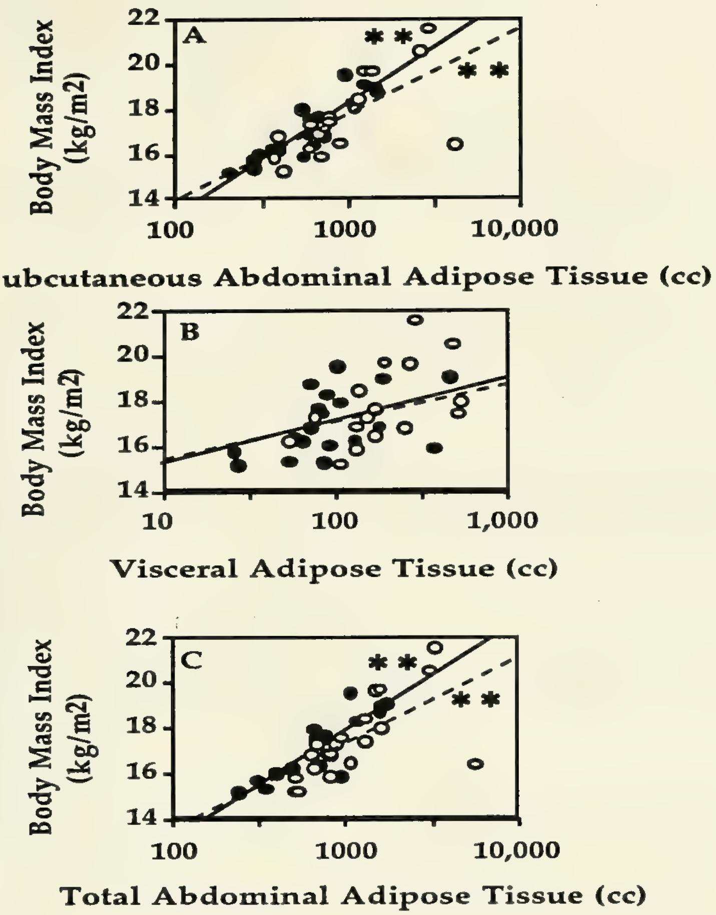
3. Metabolism

Because the onset of obesity in the majority of African American girls occurs during adolescence, we have determined the relationship of visceral fat and cardiovascular risk factors in health, normal weight, African American and Caucasian girls. We studied 20 African American and 20 Caucasian normal-weight girls ages 7-10, who were matched for weight, body mass index (BMI), bone age, chronological age, breast Tanner stage, and socioeconomic status. Each underwent abdominal magnetic resonance imaging (MRI) for determination of total (TAT), visceral (VAT), and subcutaneous (SAT) adipose tissue. Serum lipids, and both fasting and 2 hour post-glucola glucose and insulin were measured, and the correlations between risk-factors for obesity-related disorders with MRI-derived visceral, subcutaneous abdominal, and total abdominal fat content were determined.

Visceral adipose tissue, subcutaneous abdominal adipose tissue and total abdominal fat content were not significantly correlated with glucose, serum triglycerides, total cholesterol, high-density lipoprotein, low-density lipoprotein, or with systolic or diastolic blood pressure. BMI was significantly correlated with total abdominal fat ($r^2=0.72$ for African Americans, $p<0.0001$, $r^2=0.39$ for Caucasians, $p = 0.006$), and with subcutaneous adipose tissue (r^2=0.78 for African Americans, p<0.0001, r^2=0.43 in Caucasians, p=0.003), but not with visceral adipose tissue in either African Americans or Caucasians (r^2=0.19 for African Americans, p=0.06, r^2=0.10 for Caucasians, p=0.19). Both basal and 2-hour post glucola serum insulin were significantly correlated with subcutaneous abdominal adipose tissue in African American girls (r^2=0.46 for basal insulin, p=0.001, r^2=0.31 for 2h insulin, p=0.01) but not for Caucasian girls (r^2=0.03, for basal insulin ,p = 0.52; r^2=0.009 for 2h insulin, p=0.71). Insulin and total abdominal adipose tissue were also correlated in African American (r^2=0.36 for basal insulin, p=0.005, r^2=0.25 for 2h insulin, p=0.024), but not in Caucasian girls (r^2=0.02, for basal insulin ,p = 0.63; r^2=0.02 for 2h insulin, p=0.60). However, insulin was not correlated with visceral fat in either African Americans or Caucasians. VAT was not significantly correlated with any measure of insulin, or with serum lipids. However, both basal and 2-hour post glucola serum insulin were significantly correlated with SAT as assessed by MRI in African American girls (r^2=0.46 for basal insulin, p=0.001, r^2=0.31 for 2h insulin, p=0.01) but not for Caucasian girls (r^2<0.05, for basal and 2h insulin, p=NS). We concluded that there are significant racial differences in the strength of association between abdominal adipose tissue depots and insulin sensitivity in African American and Caucasian girls.



Correlations between insulin and abdominal fat depots (L1-L5) with fasting insulin (A, C, E) and with insulin obtained at 2h point of glucose tolerance test (B, D, F). Regression lines are shown for Caucasians (o-------) and African Americans (•———). * p<0.05, ** p<0.005.

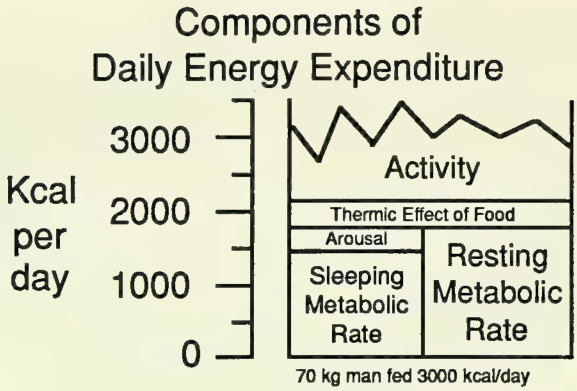


Correlations between body mass index and adipose tissue (L1-L5). Regression lines are shown both for Caucasians (o-------) and African Americans (•———). * $p<0.05$, ** $p<0.005$.

Among the girls we studied, greater quantities of visceral adipose tissue, were not associated with increases in risk factors for cardiovascular disease, including systolic blood pressure, diastolic blood pressure, total cholesterol, LDL-, or HDL-cholesterol, in either African Americans or Caucasians. This may be due to the small absolute visceral adipose tissue burden in the majority of these normal weight children. An interesting and unexpected finding was the strong association between insulin levels and both total and subcutaneous abdominal adipose tissue in African American girls, but not in Caucasian girls. A similar finding was recently reported in adults, in whom significant relationships between subcutaneous abdominal adipose tissue and both fasting insulin and insulin sensitivity, were found for African American, but not Caucasian, women. The reason for this difference in the relationship of insulin levels to the size of the subcutaneous abdominal adipose tissue depot is unknown, but could reflect racial differences in adipose tissue metabolism that are yet to be elucidated.

4. Energy Expenditure

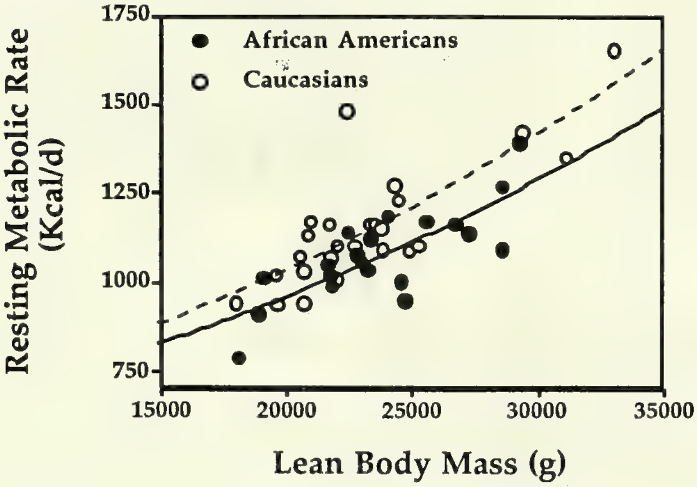
Adipose tissue deposition results when caloric intake exceeds utilization. The largest component of daily energy expenditure is the component referred to as the resting metabolic rate.



Adapted from Ravussin and Bogardus, Am J Clin Nutr 1989,49:968-75.

While resting metabolic rate (RMR) is highly correlated with fat free mass, substantial individual variance occurs, which remains unexplained by age, gender, or body composition. It has been estimated that the standard deviation of predicted RMR adjusted for fat free mass is about 124 kcal/d, which, over the long term, could substantially affect energy balance. Studies have indicated that genetic factors are important in determining the RMR, explaining about 30% of the variance, after adjustment for other known determinants. In a seminal study, Ravussin et al. found that, in 95 Pima American Indians, 24-hour energy expenditure (adjusted for body composition, age, and sex) is significantly correlated with the rate of change in body weight over a two-year follow-up period, and that the estimated risk of gaining more than 7.5 kg in body weight is increased fourfold in persons with a low adjusted 24-hour energy expenditure (200 kcal per day below predicted values) as compared with persons with a high 24-hour energy expenditure (200 kcal per day above predicted values. In another 126 subjects, the resting metabolic rate (adjusted for lean body mass, age, and sex) predicted body weight gain over a four-year follow-up period. In this latter study, when the 15 subjects who gained more than 10 kg were compared with the remaining 111 subjects, the initial mean (+/- SD) adjusted metabolic rate at rest was significantly lower in those who gained weight (1694 +/- 103 vs. 1764 +/- 109 kcal per day). These studies suggest that, at least for the adult Pima Indian population, resting metabolic rate is an important variable for predicting future weight gain.

We have investigated whether differences in resting metabolic rate might help explain the increased incidence of weight gain during adolescence in African American girls. We studied 20 African American and 20 Caucasian prepubertal girls matched for age, weight, and socioeconomic status, and determined resting metabolic rate and total lean body mass by dual energy xray absorptiometry. After correction for lean body mass, resting energy expenditure was 86 kcal/d lower in African American normal weight girls than in Caucasians (p <0.005, ANCOVA).



These data indicate that normal-weight prepubertal African American girls have reduced energy expenditure compared with Caucasian girls.

Proposed course of Project:

It is unknown whether pre-pubertal obese (or pre-obese) African American and Caucasian children demonstrate the differences in energy expenditure, insulin levels, or body composition we have found in adults and in normal weight African American and Caucasian girls.

Over the last year, a new circulating hormone, called "leptin" (from the Greek, *Leptos*, meaning thin) has been cloned in humans, and assays for determining serum levels have been produced. Leptin appears to be a very important protein made and secreted by fat cells. Mice with the *ob/ob* mutation (mice that have mutations in the sequence for leptin that inactivate the protein), become obese and diabetic, conditions that are reversed when they are given leptin by injection. The *db/db* mutant mouse and the Zucker rat, two other animal models of obesity, have recently been shown to have inactivating mutations in the leptin receptor. Leptin levels are highly correlated with percentage of body fat in adults. No data on leptin levels have as yet been published in children. There are no published data regarding circadian variation in leptin levels in adults or children, although preliminary evidence supports circadian variability in adults. At the time of this writing, no humans have been identified with leptin signalling pathway mutations.

Another exciting development in this field has been the description of a point mutation in the beta-3 adrenergic receptor, which is correlated with early onset of type-2 diabetes, greater degree of obesity, and a lower resting metabolic rate. This mutation has been described to have greater prevalence in African Americans, being present in about 12% of African Americans, in over 30% of Pima Indians, and in 8% of Caucasians.

We propose to study obese African American and Caucasian children and non-obese African American and Caucasian children of obese parents, in order to determine if racial differences in body composition, metabolic rate, insulin sensitivity, glucose disposal, leptin levels and beta-3 adrenergic receptor status exist prior to puberty, and then to follow these children longitudinally. We hypothesize that differences in these factors will predict the development of obesity in the populations studied.

Specific Hypotheses: Through this study, we will attempt to test the following hypotheses:

1. Intraabdominal adipose tissue will be highly negatively correlated with insulin sensitivity and glucose disposal in obese Caucasian children, but will account for considerably less of the variance of insulin sensitivity and glucose disposal in obese African American children. Similar results will be found in the non-obese children of obese parents.
2. Subcutaneous abdominal adipose tissue will be negatively correlated with insulin sensitivity and glucose disposal in obese African American children, but not in obese Caucasian children. Similar results will be found in the non-obese children of obese parents.
3. Resting metabolic rate (corrected for lean body mass as determined by DEXA) will be significantly lower in African American obese children than in Caucasian obese children. Resting metabolic rate (corrected for lean body mass as determined by DEXA) will predict subsequent obesity in the non-obese children of obese parents and increased degree of obesity in children who are obese at baseline. Those in the lowest quartile for resting metabolic rate will be more likely to become obese than those in the highest RMR quartile independent of race.
4. Beta 3 adrenergic receptor genotype will predict the severity and time of onset of obesity in both obese children and in children of obese parents, independent of race. Subjects with the previously described beta 3 adrenergic receptor point mutation will show increased insulin resistance and decreased glucose disposal.

5. Leptin will have significant circadian variation in children.
6. Individuals with plasma leptin levels as a function of percentage body fat in the lowest quartile at the time of initial evaluation will have greater severity of obesity, in children who are obese at initial evaluation, and an earlier time of onset of obesity in the non-obese children of obese parents, when evaluated 5, 10, or 15 years later.
7. Genotypic analysis of obese children with very high (>3 SD), or very low (<3 SD), plasma leptin levels will uncover individuals with abnormalities in the leptin signalling pathway. Such individuals will be quite rare.

Study Design and Methods:

Inclusion Criteria:

Volunteers will qualify for inclusion under this protocol if they meet the following criteria:

1. Good general health. Individuals with renal, hepatic, most endocrinologic (e.g. hypothyroidism, or Cushing syndrome), or pulmonary disorders (other than mild asthma not requiring chronic medication) will be excluded.
2. For obese subjects, body mass index for age above 85th percentile (determined by NHANES II age-, sex-, and race- specific data)
   For normal weight subjects of obese parents, BMI (adjusted for age sex and race) between 5th and 85th % and both parents' body mass index above 85th % (determined by NHANES II age-, sex-, and race- specific data). The adult 85th percentile is approximately equal to a body mass index of 27.
3. No significant psychiatric illness.
4. Tanner I (prepubertal) or Tanner II (early pubertal) pubic hair and breast stage of development for girls, and Tanner I or II pubic hair and testes size (6mL) for boys at the start of study.
5. Subjects must be able to undergo MRI. Volunteers with metal within their bodies that are contraindications for MRI will be excluded.
6. Age 6 to 10 years at the start of the study.
7. For girls who have been followed to an age when they are menstruating (or are of an age when pregnancy is a possibility), a negative pregnancy test.
8. Race of all 4 grandparents known to be either all Caucasian or all African American.

Each subject will receive a written explanation of the purposes, procedures, and potential hazards of the study. Communication of this information and of the subject's assent as well as the consent of the parent or guardian will be documented in the medical record. All participants will have the protocol explained, and sign assent/consent every 5 years, with each iteration of the protocol's procedures. All subjects will be informed of their right to withdraw from the study. All blood draws will be preceded by application of EMLA cream to the surface of the skin for a minimum of 1 hour, to anesthetize the skin surface.

Procedures of the Study:

Outpatient Evaluation

Subjects will be seen in the 9th floor outpatient clinic for a pre-admission evaluation that will include:

1. **Medical history, vital signs and limited physical exam** with Tanner pubertal staging. Assessment of growth, including weight (in kg) and height (in cm) by stadiometer (3 repeated measures) will be performed. Each child will be matched to another child (of differing race) having a body mass index within 10%.
2. **Standardized psychological tests** designed to uncover significant psychiatric illness. The Childhood Depression Inventory (CDI), Spielberger State-Trait Anxiety Scale,

Children's Eating Attitudes Test and the Child Behavior Checklist will be administered by a trained examiner. All psychological testing data will remain confidential.

3. **Evaluation of Socioeconomic Status**, determined by the Hollingshead scale. Each child will be matched another child (of differing race) within 10 Hollingshead points, to insure similar socioeconomic status. All data will remain confidential and will be reported only in the aggregate.
4. **Fasting Blood for:**
    - a. Screening for metabolic, hepatic, renal, & hematologic function: SMAC (automated blood chemistry profile), CBC (complete blood count), glycohemoglobin, cholesterol, HDL cholesterol, triglycerides, apolipoproteins, ESR (Westergren), thyroid function (15 mL)
    - b. Leptin level, IGF-1, IGF-II, GHBP, IGFBP3, (20 mL).
    - c. Sample to save for future research (20 mL).
    - d Karyotype and genomic DNA isolation (10 mL). The presence of beta-3 adrenergic receptor mutations will be determined in the laboratory of Alan Shuldiner, M.D. Leptin, leptin receptor, and other leptin signalling pathway mutations will also be studied genetically.
    - e. Pubertal assessment: estradiol, testosterone, DHT, DHEA-S, androstenedione (10 mL).
5. **Anthropometric assessment**, including measurements of skinfold thickness, total body fat using bioelectrical impedance (BIA), and body circumference using a flexible tape measure. Measurements of skinfold thickness have been found previously to be highly correlated with ponderal index and atherogenic lipid profiles in African American and Caucasian children.
5. **Bone age X-ray** as part of evaluation of pubertal development.
6. **Screening 2hr oral glucose tolerance test:**
    - a. Each child will drink 1.75 gm/kg glucose, mixed with water and decaffeinated cola syrup (standard glucola).
    - b. Two hrs later, serum glucose, insulin, and leptin level (15mL)
7. **Twenty-four hour urine collection** to be performed at home, to measure urine free cortisol and creatinine as a screen for hypercortisolism.

Inpatient Admission

Subjects will be admitted overnight to the 9-West or 11-East pediatric wards for **every 6 hour leptin levels**, for **a euglycemic and hyperinsulinemic clamp**, for measurement of **resting energy expenditure**, and for **MRI** and **DEXA** scanning for **body composition**.

Data Analysis of Study:

Primary outcomes will be the insulin and glucose values from the hyperinsulinemic and euglycemic clamp studies, body fat distribution measures obtained from skinfold thickness, body circumference, and MRI, and resting energy expenditure, corrected for lean body mass as assessed by DEXA.

Previous studies evaluating insulin levels, skinfold thicknesses, body circumferences, energy expenditure, and MRI find that measurements of energy expenditure have the highest coefficient of variation of any of the tests to be evaluated. Studies of resting energy expenditure performed at the NIH in normal weight children have indicated the standard deviation for resting metabolic rate is approximately 110 Kcal At a power level of 0.80 and an alpha level of 0.05, the sample size necessary to detect a 100 Kcal difference in the resting metabolic rate is approximately 19 in each group (one group would be African American, males). Allowing for one quarter of subjects to fail to complete the study for various reasons (e.g. inability to complete the MRI study, or failure to return for follow-up), the total number of subjects to be studied is 50 obese African American, 50 obese Caucasian, and 50 non-obese (of obese parents) African American and 50 non-obese (of obese parents) Caucasian children. To reduce background statistical noise and maximize our power to detect differences, African American and Caucasian children will be matched for socioeconomic status and for body mass index, as described above.

Between and within group differences in continuous measures will be determined through analysis of variance, with repeated measures where appropriate, followed by post-hoc tests (interpreted with correction for family error rate). Ordered and counted data will be analyzed with chi-square and Wilcoxon signed-rank test. Spearman rank will be used for correlations.

Significance to Biomedical Research and the Program of the Institute:

The increasing incidence of obesity and the earlier onset of diabetes mellitus, hypertension, and cardiovascular and renal disease which are directly related to obesity, makes understanding the mechanisms underlying weight gain in all Americans of critical importance. Obesity-related Non-insulin-dependent diabetes mellitus and its complications (such as retinopathy, atherosclerotic heart disease, nephropathy, peripheral vascular disease, and death) are 6-fold more prevalent in minority populations than in Caucasians. The staggering incidence of medically significant obesity in minority populations such as African American women (50%), and the steep increase in obesity found in children over the last 15 years makes the search for predictors of obesity and for effective prevention and treatment of the utmost value. This study, focussed on the pediatric population at particularly high risk for obesity and its complications, is thus of particular relevance for the NICHD mission.

Publications:

Yanovski JA, Yanovski SZ, Harrington L, Gold PW, Chrousos GP. Differences in the hypothalamic-pituitary-adrenal axis of African American and Caucasian men. Horm. Res. 1995; 44: 208-212.

Yanovski JA, Yanovski SZ, Cutler GB, Chrousos GP, Filmer K. Differences in the hypothalamic-pituitary-adrenal axis of African American and Caucasian girls. J Pediatr., 1996; 129:130-136.

Yanovski JA, Yanovski SZ, Friedman TC, Loh YP, Jayasvasti V, Cutler GB Jr, Chrousos GP. Etiology of the differences in corticotropin-releasing hormone-induced ACTH secretion of black and white women. J Clin Endocrinol Metab, in press.

Yanovski JA, Yanovski SZ, Filmer K, Hubbard VS, Avila N, Lewis B, Reynolds J, Flood M. Differences in body composition of African American and Caucasian prepubertal girls. Am J Clin Nutr, In Press.

ERRB

ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH

Kevin J. Catt, M.D., Ph.D., Chief

The research programs of the Endocrinology and Reproduction Research Branch (ERRB) are focused on the molecular and cellular mechanisms involved in hormone secretion and action. Major topics of interest include: the characterization of peptide hormones and their mechanisms of action in endocrine target cells; the structure-function relationships of peptide and glycoprotein hormones; and the regulation of hormone biosynthesis and secretion. At the molecular level, research is performed on the plasma-membrane receptors and intracellular signaling processes that are responsible for the control of secretory responses, differentiation, and metabolic regulation in endocrine target cells. The ERRB investigators share common interests in the mechanisms of action of peptide and glycoprotein hormones, the role of neuropeptides in hypothalamic-pituitary and gonadal regulation, the control of gonadal and adrenal function by pituitary hormones, the renin-angiotensin system and aldosterone secretion, and the mechanisms and roles of protein phosphorylation in metabolic regulation and signal transduction.

PEPTIDE HORMONES AND ENDOCRINE REGULATION

The Section on Hormonal Regulation, directed by **Kevin Catt**, performs research on the mechanisms by which peptide hormones control the activities of endocrine and other target cells. This includes the characterization of receptors, signal transduction pathways, and other cellular processes involved in the neural control of gonadotropin-releasing hormone (GnRH) biosynthesis and secretion, the regulation and structure-function properties of receptors for GnRH and angiotensin II (Ang II), and the effects of GnRH and Ang II on target cells in the hypothalamus, pituitary, and adrenal gland. Current research is focused on the structural features and signal transduction mechanisms of the GnRH and Ang II receptors, and their regulatory actions in the secretory and growth responses of hypothalamic, pituitary, and adrenal cells.

Structure-function Properties of the GnRH Receptor. The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), acts via its specific receptors in the anterior pituitary gland to regulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone, and thus plays a pivotal role in reproduction. The cDNAs for the GnRH receptors of several species, including mouse, rat, sheep, cow, and human, have recently been cloned. The deduced amino acid sequences of the receptor share more than 85% amino acid identity among species. The coding region of the GnRH receptor contains seven putative transmembrane domains (TM I-VII) as well as many of the conserved residues and sequences typical of other members of the G protein-coupled receptor (GPCR) superfamily. However, the GnRH receptor exhibits several unique features that include (a) the presence of a long and highly basic first intracellular loop; (b) the replacement of Tyr by Ser in the conserved GPCR "signature" DRY motif located at the junction of TM III and the second intracellular loop; and (c) the absence of a cytoplasmic COOH-terminal tail. Another interesting feature of the receptor is that the highly conserved Asp in TM II and Asn in TM VII are reciprocally exchanged. Upon agonist binding, the GnRH receptor activates phosphoinositide hydrolysis to diacylglycerol and inositol 1,4,5-trisphosphate, thereby mobilizing Ca^{2+} and activating protein kinase C to initiate a variety of cellular responses. Receptor activation is followed by extensive internalization and down-regulation of the receptor via endocytosis and processing. The molecular mechanism(s) of GnRH receptor activation are beginning to be clarified by structure-function studies employing site-directed mutagenesis to identify residues that are involved in G protein activation, signal transduction, and receptor internalization. In the muscarinic receptor, a conserved leucine residue in the second intracellular loop has been implicated in both signal transduction and receptor sequestration. This hydrophobic amino acid is also conserved (as Leu^{147}) in the GnRH receptor and is present at the equivalent position in the 2i loop. In studies on the structure-function relationships of the GnRH receptor, we examined the roles of the conserved Leu^{147} residue and the unique Ser^{140} residue, (which is Tyr or Trp in most other GPCRs) in agonist-induced signal transduction and receptor internalization. Replacement of Leu^{147} with either Asp or Ala impaired both G protein coupling and receptor internalization. However, replacement of Ser^{140} by Try did not affect the signaling response but increased ligand binding and receptor internalization.

In the GnRH receptor, the $\mathrm{NPX}_{2\text{-}3}\mathrm{Y}$ motif that is present in the seventh transmembrane helix of most G protein-coupled receptors is unusual in containing Asp instead of Asn, but retains the highly conserved Tyr residue. The importance of this aromatic residue in the DPLIY sequence of the GnRH receptor function was analyzed by replacing Tyr^{322} with Ala or Phe residues. The $\mathrm{Y}^{322}\mathrm{A}$ mutant receptor expressed in COS-7 cells had high agonist binding affinity, but its ability to interact with G protein(s) and to activate inositol phosphate production in response to GnRH was abolished. Although functionally inactive, the $\mathrm{Y}^{322}\mathrm{A}$ mutant receptor was internalized at about 50% of the rate of the wild type receptor in agonist-treated cells. When Tyr^{322} was replaced with Phe to preserve its aromatic nature, the $\mathrm{Y}^{322}\mathrm{F}$ mutant receptor displayed normal G protein activation and inositol phosphate responses to GnRH and was internalized in the same manner as the wild type receptor. These findings demonstrate that the aromatic moiety of the Tyr^{322} component of the DPLIY motif in the GnRH receptor is a critical determinant of agonist-induced receptor activation and signal transduction.

Regulation of Ca^{2+} Signaling and GnRH Release by Neurotransmitters. Neurotransmitters and neuropeptides modulate the secretion of GnRH from the hypothalamus and thus influence the release of gonadotrophic hormones into the circulation. Analysis of the electrophysiological effects of these regulatory factors on GnRH neurons is complicated by the small number and wide distribution of GnRH neurons in the hypothalamus, and the consequent difficulties in isolating and identifying them. The GT1-7 line of immortalized GnRH neurons has many similarities to native GnRH neurons, and has become a useful model in which to investigate the electrophysiology of the GnRH neuron. Recent studies in from GT1-7 cells have shown that the neurotransmitters, γ-aminobutyric acid (GABA) and glutamate, as well as the neuropeptides, endothelin and GnRH, increase cystoplasmic calcium ($[\mathrm{Ca}^{2+}]_i$) and GnRH secretion at least in part by depolarizing the plasma membrane, and promoting Ca^{2+} influx through L-type Ca^{2+} channels. The neuropeptides also increase $[\mathrm{Ca}^{2+}]_i$ by mobilizing Ca^{2+} from internal stores.

Calcium-activated potassium [K(Ca)] channels may be involved in dampening the excitation of GT1-7 neurons following neurotransmitter- and neuropeptide-evoked increases in GnRH release. In order for the GT1-7 cell to return quickly to a low resting $[\mathrm{Ca}^{2+}]_i$ following stimulation, the cell must undergo membrane repolarization toward its resting potential of -40 to -70 mV, followed by the closure of its voltage-sensitive Ca^{2+} channels. Delayed rectifier K^+ channels, which open in response to membrane depolarization, may mediate membrane repolarization since the outward K^+ current through the delayed rectifier K^+ channel would drive the cell toward the equilibrium potential for K^+.

K(Ca) channels, which open in response to both membrane depolarization and elevated $[Ca^{2+}]_i$ in other types, can aid in this repolarization. GT1 cells have been reported to express a variety of K^+ channels, including a delayed rectifier, an inward rectifier, a 4-aminopyridine (4-AP)-sensitive channel, and a 4-AP and tetraethylammonium (TEA)-insensitive channel. However, whether they also express K(Ca) channels has not been determined. In other cells, two types of K(Ca) channels have been characterized: the large-conductance, K(Ca), or BK, channel and the small-conductance, K(Ca), or SK, channel.

The expression and function of large-conductance Ca^{2+}-activated K^+ (BK) channels in GT1-7 neurons was investigated by measuring ionic currents using the patch-clamp technique. Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored using the flurorescent indicator, fura-2, and GnRH secretion was measured by radioimmunoassay. In cell-attached and inside-out patch-clamp recordings, K^+ channels with a single-channel conductance of ~200 pS were detected. Depolarizing the patch increased the unitary current size and the open probability. In perforated-patch recordings, depolarizing pulses (50 ms) to potentials of -10 to +60 mV from a holding potential of -90 mV elicited outward current with early transient and sustained components. The transient current peaked 2-10 ms after the beginning of each pulse and increased in a voltage-dependent manner. This current was unaffected by the small-conductance Ca^{2+}-activated K^+ channel blocker apamin (100 nM), reduced by the BK channel blocker, charybdotoxin (5 nM), abolished by the Ca^{2+} channel blocker, $CdC1_2$ (25 μM), and prolonged by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (1 μM). Based on the single-channel and whole-cell conductances, the number of channels per patch, the patch area, and the surface area of the cell, each GT1-7 cell contains 30-60 BK channels. The functional role of BK channels in GT1-7 cells was evaluated by measuring the effect of charybdotoxin (100 nM) on basal $[Ca^{2+}]_i$ and GnRH secretion, as well as on the $[Ca^{2+}]_i$ and GnRH secretory responses to γ-aminobutyric acid (GABA, 100 μM), an excitatory neurotransmitter in this system. Charybdotoxin had no effect on basal $[Ca^{2+}]_i$ or GnRH secretion, or on the GABA-evoked $[Ca^{2+}]_i$ or GnRH secretion, or on the GABA-evoked $[Ca^{2+}]_i$ and GnRH secretory responses. These results indicate that GT1-7 cells express BK channels and that the function of such channels is not related to the control of GnRH release.

Regulation of Pulsatile GnRH Release by LH Receptors Expressed in GnRH Neurons. The hypothalamic GnRH neuronal network releases GnRH in a pulsatile manner to regulate gonadotropin release from the pituitary gland, and is consequently responsible for the control of steroid hormone secretion and gamete production by the gonads. The operation of a short feedback loop between the pituitary gland and the hypothalamus has been indicated by the ability of LH to modulate its own secretion *in vivo*,and to decrease single cell firing rates in the medial preoptic area and basal hypothalamus. In addition, marked inhibition of multiunit electrical activity was observed during the initiation of the preovulatory surge of LH, coincident with the late follicular rise in serum estradiol concentration. Recent studies in cultured hypothalamic neurons, as well as immortalized pituitary gonadotrophs and GnRH-producing neurons, have begun to address the direct involvement of neuropeptides, neurotransmitters, and pituitary hormones in the control of hypothalamic GnRH secretion. Cultured hypothalamic neurons and immortalized GnRH neurons (GT1-7 cells) exhibit pulsatile GnRH release and express plasma-membrane receptors for GnRH, prolactin, and LH, as well as several other hormones and transmitters. In GT1-7 cells, agonist-induced activation of GnRH receptors leads to stimulation of phospholipase C activity, increased formation of $Ins(1,4,5)P_3$

mobilization of Ca^{2+} from intracellular stores and Ca^{2+} entry through VSCC, and modulation of GnRH release.

Treatment of GT1-7 cells with hCG caused a dose-dependent increase in cAMP production, with a rapid increase during the first 15 min and a subsequent decrease that was prevented by pre-treatment with pertussis toxin. Furthermore, the stimulatory effect of cholera toxin on cAMP production was inhibited by hCG in a dose-dependent manner. These data indicate that the LH receptors expressed in GT1-7 cells are coupled to both adenylyl cyclase stimulatory (G_s) and inhibitory (G_i) proteins. In perifused cell cultures, treatment with forskolin and 8-bromo cAMP increased the amplitude of spontaneous GnRH release. However, treatment with nanomolar concentrations of hCG abolished pulsatile GnRH release from both GT1-7 cells and rat hypothalamic cells. The similarity of hCG action on pulsatile GnRH release to that of extracellular Ca^{2+} depletion and calcium channel antagonists, and its partial resistance to potassium-induced depolarization, suggest that it results from inhibition of plasma-membrane ion channel activity. It is probable that the inhibitory action of hCG on pulsatile GnRH release is responsible for initiation of the suppression of pituitary LH secretion during pregnancy.

Expression of PAPAP Receptors in the Mammalian Ovary. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a hypothalamic neuropeptide that belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP)/growth hormone releasing hormone (GHRH) family of peptide hormones. Two biologically active forms of the PACAP molecule are derived from a single precursor protein of 176 amino acids. The larger form (PACAP-38) contains 38 amino acid residues and the smaller (PACAP-27) consists of its N-terminal 27 amino acid residues and shares 70% identity with VIP. PACAP exerts a potent stimulatory action on cyclic AMP production in anterior pituitary cells, and promotes the release of several pituitary hormones. It also stimulates phosphoinositide hydrolysis and increases cytosolic Ca^{2+} in several cell types. Like many other hypothalamic neuropeptides, PACAP and its receptors are present in a variety of extrahypothalamic tissues, including brain, testis, pituitary, respiratory system, and gastrointestinal tract. Furthermore, PACAP stimulates intracellular signaling responses and induces outgrowth of processes in human neuroblastoma cell lines.

PACAP is known to recognize two major groups of receptor sites. The type I sites have high affinity for PACAP-38 and PACAP-27 and low affinity for VIP. They are further subdivided into type IA (with equal affinity for PACAP-38 and PACAP-27) and type IB (with 100-1000 times higher affinity for PACAP-38 than for PACAP-27). In contrast, the type II sites bind PACAP 38, PACAP-27, and VIP with equal affinity. The cDNA sequence of the rat PACAP-I-R encodes a 467 amino acid protein containing the seven putative transmembrane domains that are characteristic of guanine nucleotide binding protein-coupled receptors. Five splice variants of the PACAP-I-R, with or without insertions at the C-terminal end of the third intracellular loop, have been described. These include *hip*, a 28 amino acid insert; *hop1* a different 28 amino acid insert; *hop2*, an insert similar to *hop1*, but lacking a single serine residue; and *hip-hop1*, a combination of the *hip* and *hop1* inserts. The PACAP-I-R and its isoforms are highly expressed in many areas of CNS and to a moderate extent in the pituitary gland, adrenal medulla, and testis. The ratio of PACAP-I-R to PACAP-I-*hop1*-R varies between tissues, suggesting that splicing of the PACAP-I-R gene could be differentially regulated in a tissue-specific manner.

Previous studies have demonstrated that VIP and GHRH, peptides that are structurally related to PACAP, exert biological actions on ovarian function by stimulating granulosa cells to produce cAMP, progesterone, and estrogen. PACAP has also been found to stimulate steroidogenesis and cAMP accumulation in cultured rat granulosa cells. Immunoreactive PACAP is present throughout the human female genital tract, and has been detected at low levels in the rat ovary. Expression of the PACAP gene has been observed in the fish ovary, but has not been reported in the mammalian ovary. In the rat ovary, where peptides of the PACAP/VIP/GHRH family act as local regulators of granulosa cell function, expression of the PACAP gene was demonstrated by amplification of ovarian RNA by the reverse transcription/polymerase chain reaction (RT-PCR). In addition, hybridization of Northern blots of rat ovarian poly(A)$^+$ RNA with a 706-nt rat hypothalamic PACAP-I receptor cDNA probe revealed the presence of a 7.0 kb PACAP receptor transcript, similar to that detected in brain and hypothalamus. RT-PCR using specific primers for the PACAP-I-R gene yielded products of the expected size with RNA obtained from ovarian tissue, brain, and hypothalamus. The authenticity of the PCR products was confirmed by Southern blotting and nested PCR, which revealed at least three splice variants of the PACAP-I-R in the rat ovary. These findings demonstrate that both PACAP and PACAP-I receptor isoforms are expressed in the rat ovary, where they could exert autocrine and/or paracrine actions on granulosa cell function.

Autocrine Actions of Endothelin in Ovarian Cancer Cells. The identification of endothelin as a potent vasoconstrictor peptide produced by vascular endothelial cells was followed by the recognition of a family of 21-amino acid isopeptides, termed ET-1, ET-2, and ET-3. Endothelins exert their biological actions through at least two major receptor subtypes, ET_A and ET_B, that are defined by their relative affinities for ET-1, ET-2, and ET-3. ET_A receptors are characterized by high and equal affinity for ET-1 and ET-2, and 100-fold lower affinity for ET-3, whereas ET_B receptors display high and similar affinity for all three endothelin peptides. ETs and sarafotoxins initiate a common signal transduction pathway, principally through G protein-mediated activation of phospholipase C and subsequent inositol trisphosphate-dependent increases in intracellular Ca^{2+} levels.

ET peptides and their receptors are expressed in numerous tissues and cell types, including the cardiovascular and nervous systems, and appear to be involved in a wide variety of physiological functions. In addition to activating phosphoinositide hydrolysis and calcium signaling, ET-1 stimulates mitogenic and proliferative responses in vascular smooth muscle and other cell types, as well as the rapid expression of *c-fos* and *c-myc*. These and other findings have demonstrated that ET-1, like several other calcium-mobilizing hormones, has the capacity to elicit growth responses in its target tissues. Recently, ET-1 has been found to be produced in a number of neoplasms, including laryngeal, lung, hepatocellular, pancreatic, colon, mammary, and cervical carcinomas. In conjunction with observations that ET-1 has TGF-β-like activity on the anchorage-independent growth of NRK 49F cells, its production by a variety of tumor cells has led to the proposal that ET-1 may participate in the pathogenesis of certain types of cancer through an autocrine/paracrine mechanism. Recent studies on ovarian cancer, which is a major cause of death from gynecological malignancy, have focused on factors that regulate the growth of malignant ovarian epithelium. A more fundamental understanding of these factors might suggest novel and potentially more effective approaches to the treatment of ovarian neoplasms. Significant amounts of ET-1 mRNA are present in the bovine ovary, and immunoreactive ET-1 has been detected in culture medium of porcine granulosa cells and in ovarian follicular fluid.

When the production of ET-1 and its receptor-mediated actions on calcium signaling and growth responses were analyzed in human ovarian carcinoma cells, immunoreactive ET-1 was produced by three of four ovarian tumor cell lines. Reverse-phase HPLC and radioimmunoassay of conditioned media from tumor cells revealed a single peak coeluting with authentic ET-1. The ET-1 producing cell lines also expressed high-affinity ET_A receptors (K_d<0.1 nM) that ranged in abundance from 2,600 to 43,600 sites/cell. In fura-2-loaded ovarian carcinoma cells, ET-1 induced dose-dependent increases in cytoplasmic Ca^{2+} concentration. ET-1 also stimulated thymidine incorporation in the three cell lines that expressed ET receptors. The ET_A receptor antagonist, BQ 123, inhibited the stimulatory actions of ET-1 on thymidine incorporation and cell proliferation in OVCA 433 cells, and substantially reduced the basal growth rate of unstimulated ovarian tumor cells. These results demonstrate that ET-1 is produced in ovarian cancer cells and acts as an autocrine growth factor by binding to ET_A receptors and stimulating calcium signaling and proliferative responses. Such findings suggest that ET-1 participates in the progression of neoplastic growth in certain ovarian tumors.

Structure-function Studies on AT_1 Receptors: Since the cloning of the Ang II receptors in 1992, efforts have been made to identify structural features, specifically amino acid residues and sequences, that are involved in the processes of ligand binding, agonist activation and G protein coupling, and internalization of agonist-receptor complexes. However, much remains to be discovered about the individual residues involved in these processes, and the intramolecular interactions between specific regions of the receptor that lead to the conformational changes necessary to adopt the active conformation of the receptor. This problem applies not only to the Ang II receptors but to all members of the G protein-coupled (GPCR) superfamily. In previous studies the amino acid residues that form the binding site for the clinically important non-peptide antagonist, losartan, were found to be located exclusively within the transmembrane domains of the receptor. In addition, residues within the cytoplasmic tail of the receptor that are essential for agonist-induced internalization of the ligand-receptor complex have been identified. Current studies have focused on the identification of residues that are involved in the Ang II-induced activation of the receptor, and its coupling to the plasma-membrane effector systems that mediate intracellular signaling responses.

The AT_1 receptor interacts with several G proteins, including G_q, G_i, and G_o, but its major physiological functions are expressed through G_q-mediated activation of phospholipase C, followed by phosphoinositide hydrolysis and Ca^{2+} signaling. Although all GPCRs possess the basic seven-transmembrane structure, only a few amino acids highly conserved among this large superfamily of receptors. In most GPCRs for peptide hormones, the binding site for physiological agonists is formed by amino acids located in superficial regions of the intramembrane helices and the extracellular regions of the receptor. The conformational change evoked by agonist binding is transmitted by the transmembrane helices to the intracellular loops, which serve to couple the receptor to its cognate G protein(s) and thence to intracellular signaling systems. Mutagenesis and biochemical studies have demonstrated that the amino-terminal portion of the third intracellular loop is a major structural element involved in receptor activation and G protein recognition. Secondary structure predictions suggest that this region is an amphiphilic α-helical extension of the fifth transmembrane helix. In accordance with the proposed importance of this region in G protein activation, synthetic peptides corresponding to the N-terminal portion of the third intracellular loop can activate the appropriate G proteins *in vitro*. However, relatively few studies have identified specific amino acids that are required for receptor activation, and the structural determinants of the G protein coupling specificities of GPCRs are still unclear.

Site-directed mutagenesis studies with muscarinic, β-adrenergic, formyl peptide, and Ang II receptors have found no evidence that charged residues in this amphiphilic segment participate in G protein coupling. However, uncharged amino acids in this portion of the third intracellular loop may be important determinants of G protein recognition and activation. One such residue is Tyr^{254} in the M_3 muscarinic receptor, which is conserved between muscarinic receptors that activate phosphoinositide signaling (M_1, M_3 and M_5) but is replaced by a serine residue in those (M_2 and M_4) that inhibit adenylyl cyclase. However, most of the GPCRs that stimulate phosphoinositide turnover do not possess a tyrosine residue in this position. Identification of other amino acids that determine G protein coupling is important in providing clues about the nature of the interaction between the receptor and its G proteins(s), and the mechanisms of G protein activation and specificity.

As noted above, the amino-terminal portion of the third intracellular loop is a critical region for G protein coupling of the rat AT_{1a} receptor. In addition, substitution of phenylalanine for a conserved tyrosine residue (Tyr^{215}) in the fifth transmembrane domain, near the origin of the third cytoplasmic loop, caused loss of AT_{1a} receptor activation. Analysis of the amino acids in this region of the AT_1 receptor identified Leu^{222} as an essential residue in receptor activation by Ang II. Non-polar replacements for Leu^{222} yielded functionally intact AT_1 receptors, while polar or charged residues caused progressive impairment of inositol phosphate generation without affecting receptor affinity for the peptide antagonist, $[Sar^1,Ile^8]$Ang II. The decrease in Ang II-induced signal generation was associated with a parallel reduction of receptor internalization, and was most pronounced for the Lys^{222} mutant receptor. Although this mutant showed normal antagonist binding, its affinity for Ang II was markedly reduced, indicating that the Lys^{222} receptor is unable to adopt the high affinity conformation. A search revealed that many G_q-coupled receptors contain an apolar amino acid (frequently leucine) in the position corresponding to Leu^{222} of the AT_{1a} receptor. These findings demonstrate that this conserved residue in the third intracellular loop is a key element in the agonist-induced activation of the AT_{1a} receptor.

Properties of AT_1 Receptor Subtypes Expressed in Adrenal Y-1 Cells. Studies on the pharmacology and functional properties of Ang II receptors in various tissues and cells have revealed significant heterogeneity of the receptors in terms of their size, second messenger systems, and sensitivity to reducing agents. Recently, the use of novel peptide and nonpeptide antagonists has demonstrated the existence of two major subtypes of the Ang II receptor, AT_1 and AT_2, and has clarified some of the earlier differences observed between Ang II receptors in certain tissues. Most of the known regulatory functions of Ang II in its target cells have been found to be mediated by AT_1 receptors. However, signaling processes regulated by AT_2 receptors, including regulation of ion channels and phosphotyrosine phosphatase activity, have been recently identified.

The cloning of AT_1 receptors from vascular smooth muscle and bovine adrenal cells has more completely defined the molecular basis of Ang II receptor heterogeneity. Two closely related forms of the AT_1 receptor have been identified in the rat and mouse. These exhibit marked DNA sequence homology in their coding regions but differ significantly in their noncoding regions and in their relative tissue distributions. These AT_1 receptor subtypes, termed AT_{1a} and AT_{1b}, are present in most Ang II-responsive tissues in varying proportions, which complicates the analysis of their binding pharmacology and coupling to intracellular effector systems. Studies on the properties of the two AT_1 receptor subtypes expressed in COS-7 cells and in *Xenopus laevis* oocytes have suggested differences in their ligand selectivity and regulation of Ca^{2+} signaling. A more complete characterization of the rat AT_{1a} and AT_{1b} receptor subtypes was obtained in stably transfected adrenocortical Y-1 tumor cells.

Selected clones of transfected cells expressing the AT_{1a} or AT_{1b} receptor subtypes bound the native ligand Ang II and the peptide antagonist [Sar^1,Ile^8]Ang II with similar affinities, but differed in their relative affinities for the nonpeptide antagonist losartan, and the peptide antagonist [Sar^1,Gly^8]Ang II. Photoaffinity labeling of the expressed receptors revealed a single 65 kDa component for both receptor subtypes, suggesting that they are glycosylated in a similar manner. Ang II stimulated increases in inositol phosphate formation and cytoplasmic Ca^{2+} in both AT_{1a}- and AT_{1b}-transfected Y-1 cells in a pertussis toxin-insensitive manner. However, Ang II failed to inhibit agonist-stimulated cAMP production, in contrast to its inhibitory action on adenylyl cyclase in several Ang II target tissues. These findings indicate that AT_{1a} and AT_{1b} receptors show small but significant differences in their binding pharmacology and, upon activation, are coupled through G_q/G_{11} to the phosphoinositide-Ca^{2+} signaling pathway. However, neither of the AT_1 receptor subtypes exhibits coupling to G_i and inhibition of adenylate cyclase when expressed in murine adrenal tumor cells or COS-7 cells.

A Novel Phosphatidylinositol 4-kinase Essential for Agonist-induced Ca^{2+} Signaling. The fungal metabolite, wortmannin (WT), a potent inhibitor of myosin light chain kinase, was previously found to prevent the sustained but not the initial increases in $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ in Ang II-stimulated adrenal glomerulosa cells. The inhibition of Ca^{2+} influx by WT was shown to be a consequence of reduced $Ins(1,4,5)P_3$ formation, and could reflect the resultant refilling of the agonist-sensitive Ca^{2+} pool. Further exploration of the site of action of WT focused on the pathway responsible for biosynthesis and maintenance of phosphatidylinositol 4,5-bisphosphate [$PtdIns(4,5)P_2$] the immediate precursor of intracellular signals generated by calcium-mobilizing hormones and growth factors. The synthesis of $PtdIns(4,5)P_2$ is initiated by the conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate [PtdIns(4)P] by phosphatidylinositol 4-kinase (PtdIns 4-kinase). Most cells contain several PtdIns 4-kinases, but the enzyme responsible for regulating the synthesis of hormone-sensitive $PtdIns(4,5)P_2$ pools has not been identified.

Micromolar concentration of WT inhibited the synthesis of hormone-sensitive PtdIns(4)P and $PtdIns(4,5)P_2$ pools in intact adrenal glomerulosa cells, and a WT-sensitive PtdIns 4-kinase was identified in adrenocortical extracts. In addition to its sensitivity to the PtdIns 3-kinase inhibitor WT, this enzyme is distinguished from the recognized membrane-bound PtdIns 4-kinases by its molecular size and weak membrane association. Inhibition of this PtdIns 4-kinase by WT results in rapid loss of the hormone-sensitive $PtdIns(4,5)P_2$ pool in angiotensin II-stimulated glomerulosa cells. Consequently, WT treatment inhibits the sustained but not the initial increases in inositol 1,4,5-trisphosphate and cytoplasmic $[Ca^{2+}]$ in a variety of agonist-stimulated cells, including adrenal glomerulosa cells, NIH 3T3 fibroblasts, and Jurkat lymphoblasts. These findings indicate that a specific WT-sensitive PtdIns 4-kinase is critical for the maintenance of the agonist-sensitive polyphosphoinositide pool in several cell types.

While the role of PtdIns(4,5)P_2 in intracellular signaling is well recognized, it has become evident that polyphosphoinosides are also involved in the regulation of more diverse cellular functions. Actin binding proteins such as profilin have been shown to bind PtdIns(4,5)P_2, and this interaction has a significant impact on the ability of phospholipase C isozymes to hydrolyze the lipid. Adaptor proteins that participate in clathrin-coated pit assembly (and hence receptor-mediated endocytosis) also bind PtdIns(4,5)P_2 (as well as some highly phosphorylated inositol derivatives), and such interactions affect their function. More recently, the critical role of PtdIns(4,5)P_2 in the G nucleotide-dependent activation and association of phospholipase D with various small G proteins (ARFs and Rho) has been the focus of numerous studies. Thus, the clarification of pathways leading to phosphoinositide synthesis has implications for several aspects of phospholipid-regulated cell signaling.

In contrast to these multiple functions of PtdIns(4,5)P_2, the manner in which 3-phosphorylated inositol phospholipids influence growth responses and other important cell functions has yet to be defined. The 3-phosphorylated inositides represent only a small fraction of the total phosphoinositide population and are produced by PtdIns 3-kinases. These enzymes are activated by receptor tyrosine kinases as well as by oncogenic tyrosine kinases, and are important for several aspects of cell regulation. Since most of the known actions of phosphoinositides require the phosphorylation of PtdIns, the phospholipid kinases are essential participants in the regulation of multiple cell functions. Although the importance of 4-phosphorylated inositides was recognized much earlier than that of the 3-phosphorylated forms, considerably more is known about PtdIns 3-kinases than about PtdIns 4-kinase. The first PtdIns 3-kinase to be isolated and cloned has 110 kDa catalytic and 85 kDa regulatory subunits, and phosphorylates PtdIns and PtdIns-(4)P as well as PtdIns(4,5)P_2. Additional 3-kinases have been described recently, including a PtdIns-specific 3-kinase that phosphorylates only PtdIns but not its phosphorylated derivatives, and others that are regulated by G protein $\beta\gamma$ subunits. These enzymes are soluble and can be inhibited by wortmannin, a fungal metabolite, with potencies that vary among the individual forms of the enzyme.

Since PtdIns4-kinase activity is most abundant in the membrane fraction of homogenized tissues and can be solubilized by detergents, most attempts to purify the enzyme have utilized the membrane-bound forms. Two forms of detergent-soluble PtdIns 4-kinase have been identified: type II, a ~56 kDa adenosine-sensitive enzyme; and type III, a larger (>200 kDa) form that is less sensitive to inhibition by adenosine. The enzymatic activity of adrenal cPI4K was inhibited not only by WT but also by LY-294002, another inhibitor of PtdIns 3-kinase, and neither compound affected type II PtdIns 4-kinase at concentrations that inhibited cPI4K. In contrast to the type II enzyme, cPI4K had a significantly higher K_m for ATP, was relatively insensitive to inhibition by adenosine ($K_i \sim 800\ \mu M$ vs $40\ \mu M$), had lower affinity for PtdIns, and was not inhibited by Ca^{2+} ions. These properties identify the WT-sensitive adrenal cPI4K as a type III PtdIns 4-kinase that is distinct from the tightly membrane-bound, Ca^{2+}- and adenosine-sensitive, type II PtdIns 4-kinase. The kinetic parameters of the type III PtdIns 4-kinase from bovine brain were similar the adrenal cPI4K, and was also inhibited by WT with an IC_{50} of 30-50 nM. Since WT inhibits the synthesis of agonist-regulated phosphoinositide pools in intact cells at micromolar concentrations, these findings indicate that type III rather than type II PtdIns 4-kinases are responsible for the maintenance of the precursor phospholipids required for intracellular signaling through the inositol phosphate/Ca^{2+} pathway.

Calcium Signaling in Adrenal Glomerulosa Cells. Repetitive Ca^{2+} transients occur in a variety of cell types following stimulation with low concentrations of Ca^{2+}- mobilizing agonists, and consist of a series of Ca^{2+} spikes that occur at intervals of 0.5 to 5 min, depending on the agonist dose and the cell type. Such oscillatory signals often persist for 1 hour or more, and have been observed in cell types ranging from electrically non-excitable cells such as hepatocytes, through partially excitable endocrine and neuroendocrine cells (e.g., pancreatic β cells, pituitary cells, and adrenal glomerulosa cells) to classical excitable cells (muscle cells, neurons). In many cases the spiking frequency of the oscillations is regulated by the agonist concentration, providing the basis of a frequency-encoded Ca^{2+} signaling mechanism. Although Ca^{2+} release from internal stores is a major source of the cytoplasmic Ca^{2+} transients, $[Ca^{2+}]_i$ levels are also influenced by Ca^{2+} entry mechanisms operating through plasma membrane Ca^{2+} channels. These include a ubiquitous form of Ca^{2+} influx in which the filling state of the Ca^{2+} stores regulates Ca^{2+} influx ("capacitative" Ca^{2+} entry), as well as various types of voltage-sensitive calcium channels (VSCC) that regulate Ca^{2+} influx as a function of the membrane potential in excitable cells.

In many cell types, the generation of Ca^{2+} oscillations depends on the dual regulation of the $Ins(1,4,5)P_3$ receptor channel by the ambient cytosolic Ca^{2+} concentration, with positive and negative feedback by low and high $[Ca^{2+}]_i$ levels, respectively. This mechanism has been proposed to initiate and terminate short bursts of Ca^{2+} release even at constant $InsP_3$ levels. The restoration of basal $[Ca^{2+}]_i$ between spikes depends on diverse Ca^{2+} homeostatic mechanisms, including Ca^{2+} pumps located in the endoplasmic reticulum (ER) and plasma membrane (PM). These processes, and possibly uptake by mitochondria, re-establish the resting $[Ca^{2+}]_i$ levels and also refill the Ca^{2+} pools so that a new cycle can be initiated. Due to the high Ca^{2+} sensitivity of the $Ins(1,4,5)P_3$ receptor channel, changes in the Ca^{2+} homeostatic mechanism(s) of the cell affect the rate and character of Ca^{2+} oscillations. For example, the Ca^{2+} wave frequency in *Xenopus* oocytes is accelerated by enhancement of Ca^{2+} influx, as well as by overexpression of a SERCA type Ca^{2+} pump to promote the rapid resetting of basal $[Ca^{2+}]_i$ levels. These findings indicate the involvement of both Ca^{2+} entry and reuptake mechanisms in the regulation of periodic calcium signaling in these large cells. Recent microfluorimetric studies revealed that small microdomains (or hot spots) with little impact on the average $[Ca^{2+}]_i$ of the cell may have a crucial role in the regulation of intracellular Ca^{2+} signaling events. This implies that if the plasma membrane Ca^{2+} entry channels are adjacent to the Ca^{2+} storage sites with their $Ins(1,4,5)P_3$ receptors, the local actions of even small amounts of Ca^{2+} infux through such channels could be important in the regulation of intracellular Ca^{2+} signals.

The adrenal glomerulosa cell possesses both the capacitative Ca^{2+} entry pathway and various types of VSCC. The activation of these Ca^{2+} influx mechanisms during Ang II stimulation provides two complementary mechanisms to regulate intracellular Ca^{2+} signaling through Ca^{2+} entry. Previously, blockade of VSCC by dihydropyridines was found to cause minor but consistent inhibition of the $[Ca^{2+}]_i$ plateau response elicited by high doses of Ang II in suspensions of adrenal zona glomerulosa cells. Such results suggested that voltage-dependent Ca^{2+} channels make relatively little contribution to the Ca^{2+} signals generated by Ang II in adrenal cells. However, the aldosterone secretory response of these cells is reduced by dihydropyridines, especially at low Ang II concentrations. This apparent discrepancy could be resolved if the role of VSCC activation is not to increase the overall cytosolic Ca^{2+} levels but rather to modulate Ca^{2+} oscillations that occur primarily at lower, more physiological Ang II levels. The relationships between Ca^{2+} influx pathways and the Ca^{2+} release process that is the basis of Ca^{2+} oscillations were analyzed in Ang II-stimulated bovine glomerulosa cells. Ang II

(100 pM) induced repetitive $[Ca^{2+}]_i$ spikes, the frequency of which was regulated over a narrow range of Ang II concentrations. In the absence of externally added Ca^{2+}, Ca^{2+} oscillations were maintained for prolonged periods of time but their frequency was significantly lower than in the presence of Ca^{2+}. Elevation of $[Ca^{2+}]_e$ from zero to 0.6 mM increased the frequency of Ca^{2+} oscillations but only in cells that showed narrow spikes with undiminishing amplitude. However, in cells that responded with broad spikes of markedly decreasing amplitude, addition of Ca^{2+} changed the oscillations to a sustained plateau of $[Ca^{2+}]_i$. In the presence of Ca^{2+}, the frequency of the oscillatory Ca^{2+} response to 100 pM Ang II was reduced by nifedipine and was accelerated by BAY K 8644, or shifted to a sustained $[Ca^{2+}]_i$ plateau normally evoked by higher Ang II concentrations (>1 nM). These findings indicate that Ca^{2+} influx through VSCCs modulates the frequency of synchronized Ca^{2+} oscillations induced by low agonist concentrations, by a mechanism that does not involve major changes in inositol trisphosphate formation. In contrast, VSCCs make relatively little contribution to the non-oscillatory Ca^{2+} signals generated by high agonist concentrations, when Ca^{2+} influx occurs primarily through the capacitative Ca^{2+} entry pathway.

Cloning and Expression of a Mammalian Adrenomedullin Receptor. The potent hypotensive peptide, adrenomedullin (AM), was originally isolated from a human adrenal pheochromocytoma using a detection system based on its ability to elevate platelet cAMP levels. Subsequent work revealed that this peptide is produced by a wide variety of tissues, most notably the adrenal, lung, kidney, and heart. It is also synthesized and secreted in abundance by vascular endothelial cells and is detectable in the circulation. The principal physiological action of AM appears to be as a potent vasodilator, and its systemic administration causes a rapid and profound fall in blood pressure and an increase in pulmonary blood flow. AM has also been found to act as a bronchodilator, an inhibitor of drinking behavior, and an inhibitor of angiotensin-induced aldosterone secretion.

There has been difficulty in defining the receptor sites of AM, and several studies have suggested that its actions are mediated through calcitonin gene-related peptide (CGRP) receptors. There is modest sequence homology between these peptides, in particular the presence of a six residue intramolecular disulfide-linked ring structure that is also present in islet amyloid polypeptide. CGRP has similar vasodilator properties to AM, and this action of AM can be inhibited by the CGRP antagonist, CGRP[8-37]. However, while the specific AM biding sites in rat vascular smooth muscle cells exhibit a K_d of 13 nM, the K_i for CGRP at these sites (300 nM) was significantly greater than that for binding to the CGRP receptor, suggesting that specific receptors for AM do exist and have low affinity for CGRP. Identification of a cDNA clone for such an AM receptor (AM-R) would enhance our understanding of the physiology of this peptide, and facilitate the development of therapeutically useful drugs that could stimulate or block these receptors.

A cDNA clone that was originally isolated as an orphan receptor from rat lung during a search for AT_1 receptor cDNAs was found to encode the adrenomedullin receptor. The receptor is a polypeptide of 395 residues that contains seven transmembrane domains and has a general structural resemblance to other members of the G protein-linked receptor superfamily. When expressed in COS-7 cells, the receptor mediates a cAMP response to adrenomedullin with an EC_{50} of 7 nM, and binds ^{125}I-adrenomedullin with a K_d of 8.2 nM, properties that are consistent with those observed in cardiovascular and other AM target tissues. The receptor gene is expressed as several mRNA species, the most prominent of which is a 1.8-kilobase transcript found in the lung, adrenal, heart, spleen, cerebellum, and other sites. Identification of this receptor cDNA should facilitate further investigation of the cellular actions of adrenomedullin and its regulatory effects in normal and disordered states of cardiovascular function, and in the development and growth of non-vascular cell types.

STRUCTURE AND FUNCTION OF PEPTIDE HORMONES AND THEIR RECEPTORS

This project, supervised by **Hao-Chia Chen**, is concerned with the analysis, synthesis, and structure-function relationships of biologically active peptides and proteins and their receptors with particular emphasis on the study of structural and physico-chemical characteristics in order to elucidate structural requirements for various biological activities. Currently, the primary research objectives are to analyze the structure and function of angiotensin II (AII) and gonadotropin releasing hormone (GnRH) receptors. In the past year, advances have been made in two major areas: (1) chemical synthesis of peptide fragments derived from protein sequences of the two receptors, and (2) high yield expression of full length and partial sequences of the receptor molecules.

Expression of the Rat Angiotensin AT_1 Receptor. Following the previous production of the carboxyl-terminal receptor segment (designated as LE92, corresponding to residues 268-359 of the rat AT_{1b} receptor) in bacterial expression systems, the full-length receptor and its 1-240 residue segment were expressed in baculovirus/Sf9 insect cell systems as non-fused and glutathione-S-transferase fusion proteins. A vector containing 1.3 kb of the AT_{1b} receptor cDNA was used to obtain 1.08 kb and 0.72 kb gene sequences. The 1.08 and 1.3 kb cDNA sequences were cloned into the pVL1393 plasmid, resulting in the expression of a non-fused protein, and the 1.08 and 0.72 kb sequences were cloned into the glutathione transferase (GST) plasmids, pAcG2T and pAcSecG2T. The latter construct has an in-frame gp67 signal sequence preceding the GST gene to promote secretion of the fusion protein. Western blot analysis of the expressed proteins, using an antiserum raised against the C-terminal LE92 segment, revealed bands ranging from 66-210 kDa. The recombinant proteins were found largely in the particulate fraction (presumably in the inclusion bodies) and none was detected in the soluble fraction or medium, even when the secretory signal sequence was present in the vector. Ang II binding activity was detectable but was lower than that of partially purified bovine adrenal membranes Similar results were obtained from the lysate of cells transfected with the 1-240 receptor segment, using an antiserum against the 1-26 residue N-terminal peptide. Purification of these GST fusion proteins by glutathione affinity chromatography had only limited success. The combination of high receptor protein levels and poor Ang II binding activity suggested that the majority of the expressed protein was in the inclusion bodies, and that denaturants would be necessary to extract and refold the protein to generate an active form. Since such conditions would preclude the use of GST affinity chromatography to purify the protein, the "Histag" system was employed in further studies as described below.

High-Yield Expression of the Human AT_1 Receptor. Improved baculovirus protein expression systems, including the recombinant viral plasmid pFastBac 1 and insect host cells from the cabbage looper *(Trichoplusia ni)*, were utilized for expression of the AT_1 human receptor. Studies on the human receptor have the advantage of potential clinical application, in addition to gaining basic knowledge in receptor biology. Unlike the rodent species, only a single AT_1 receptor is present in the human. A gene encoding the human AT_1 receptor (hATR1-PcDNA I, SmithKline Beecham, Inc.) was used to construct a recombinant donor plasmid in the pFastBacHTb plasmid, where the AT_1

sequence is preceded with six consecutive histidines (6XHis), a spacer, and an rTEV protease cleavage site. When the recombinant bacmid DNA generated from *E. coli* (*Lac7*$^{-}$) was transfected into insect cells, radioligand binding analysis of the optimally infected cells detected 90 times more binding sites than those found in the bovine adrenal membranes. This level of expression is a significant improvement over all known natural sources. However, the majority of the receptor protein was again found in the particulate fraction, as revealed by Western blot analysis using antiserum against the LE92 fragment of the rat AT_{1b} receptor, which revealed, multiple bands ranging from 66-210 kDa. Although the receptors are still predominantly intracellular, there is sufficiently abundant expression at the plasma membrane to permit further purification by Ni-affinity chromatography after solubilization in guanidine chloride. This work is now in progress, and large scale production of the His-tagged protein has been initiated.

Chemical Synthesis and High-Yield Expression of GnRH Receptor Segments. Based on the deduced amino acid sequence of the GnRH receptor, seven segments from different domains in the receptor protein were selected and synthesized by the solid phase method. These included residues 316-327 in the putative seventh transmembrane domain and the C-terminus; residues 248-266 in the third cytoplasmic domain; residues 14-35 in the first extracellular domain; residues 229-270, the entire third cytoplasmic domain; residues 178-197 in the third extracellular domain; residues 291-306, the fourth extracellular domain; and residues 105-121 & 186-198, the second and third extracellular domains cross-linked between two inner Cys residues. Two more peptides from the cytoplasmic domain were also synthesized, residues 58-82 and 134-158, respectively. The peptides were conjugated to thyroglobulin through Sulfo-SMCC, a heterobifunctional linker, via added cysteine on the N-terminus of the peptide. Rabbits immunized with these conjugates produced appreciable antibody titers. However, the anti-peptide antibodies were of low avidity lacked sufficiently specificity for the immunological characterization of the receptor protein. In order to generate antibodies of higher affinity and selectivity, larger sequences of the receptor were expressed as segments containing the extracellular, transmembrane, and cytoplasmic domains. By this approach, milligram quantities of three maltose-binding protein (MBP) fusion proteins containing the receptor sequences corresponding to amino acid residues 19-77, 138-194, 292-327, and 265-359 were obtained. These sequences were expressed in *E. coli* transfected with a plasmid containing the corresponding gene inserted downstream from the *mal*E gene in the pMAL-2c vector, which also contains a sequence encoding the recognition site of the specific protease Factor Xa located at the 5' insertion site. The expression of this construct results in the production of an MBP fusion protein that allows one-step purification by maltose-affinity chromatography. Purification of fusion proteins from the cell lysate by maltose-affinity chromatography, and cleavage of the fusion proteins with Factor Xa, yielded fragments of the expected sizes as revealed on SDS-PAGE. Under optimal conditions, more than 75% efficiency of cleavage was obtained without generating other potential internal cleavage products. These MBP-receptor fusion proteins are being used to immunize rabbits for the generation of receptor-specific antibodies.

INTRACELLULAR SIGNALING IN ENDOCRINE CELLS

The pituitary gonadotroph has proven to be an excellent model for the study of calcium homeostasis in excitable cells. **Stanko Stojlkovic** and his colleagues in the Unit on Cellular Signaling have used these cells to advantage in their continuing studies on plasma membrane- and endoplasmic reticulum-derived calcium oscillations and calcium-controlled cellular functions. The current emphasis in these investigations is on the integration of voltage-sensitive and -insensitive calcium entry pathways in gonadotropin-releasing hormone (GnRH)-induced and $InsP_3$-mediated calcium oscillations, and in luteinizing hormone (LH) release by gonadotrophs.

Modulation of $InsP_3$-Induced $[Ca^{2+}]_i$ Oscillations by Voltage-Gated Calcium Entry. The influence of sustained changes in the rate of Ca^{2+} entry on the pattern of agonist/InsP3-induced Ca^{2+} spiking was investigated in collaboration with Drs. Leoncio Vergara and Manuel Kukuljan, University of Chile. In these studies, nystatin-perforated and whole-cell modalities of the patch-clamp technique were employed to permit rapid and transient modulation of calcium entry without affecting the $[Ca^{2+}]_i$ by diffusion from the pipet, and for injection of $InsP_3$, respectively. In current-clamped cells, the frequency of the GnRH- and $InsP_3$-induced oscillations increased with time, while the amplitude decreased until both reached stable values. However, when plasma membrane protential (V_m) was held at -50 mV or below, both parameters progressively decreased until the signal was abolished. These effects were reversed by depolarizing the membrane positive to -45 mV in both agonist- and $InsP_3$-stimulated gonadotrophs. Depolarization also led to an increase in the fraction of time during which $[Ca^{2+}]_i$ remained elevated. This effect originated both from an increase in the duration of spikes (time-on) and a decrease in the interspike periods (time-off). The frequency and amplitude of spiking, as well as the on and off times, depended on the activity of voltage-sensitive calcium channels, but displayed different temporal courses and voltage relationships. The depolarization-driven recovery of the frequency was instantaneous, while the recovery of the amplitude of spiking was more gradual. The midpoints of the V_m sensitivity curve for time-on and amplitude of spiking were close to the values observed in the L-type Ca^{2+} current and in the depolarization-induced increase in $[Ca^{2+}]_i$, while this parameter was much lower for time-off and frequency of spiking. These observations indicate that Ca^{2+} entry exhibits two distinct positive feedback effects on $InsP_3$-evoked Ca^{2+} release. It facilitates Ca^{2+} liberation due to its direct and instantaneous action on the release mechanism, and also magnifies the Ca^{2+} signal due to its gradual effect on the balance between Ca^{2+} efflux and influx.

Expression of Purinergic Receptor Channels in Pituitary Cells. Adenosine trisphosphate (ATP) is frequently stored in secretory granules together with other neurotransmitters and hormones, and is often co-secreted during agonist stimulation. Once released into the extracellular medium, ATP may bind to (P_2 purinergic receptors in the plasma membrane, leading to increases in $[Ca^{2+}]_i$ and potentiation of exocytosis. ATP receptors belong to two major groups: G protein-coupled receptors (P_{2Y}), which mediate Ca^{2+} release from its intracellular stores, and plasma membrane receptor-channels (P_{2X}) that promote Ca^{2+} influx. Pituitary gonadotrophs were found to express P_{2X} receptor-channels, which are activated by ATP and ADP, but not by AMP or adenosine. In identified gonadotrophs, ATP induced dose- and extracellular Ca^{2+}-dependent elevations of $[Ca^{2+}]_i$ in an Mg^{2+}- and suramin-sensitive manner. ATP, adenosine-5'-0-(3-thiotriphosphate), adenosine-5'-0-(1-thiotriphosphate), 2-methylthio-ATP, and 3'-0-(4 benzoyl)benzoyl-ATP were roughly equipotent in elevating $[Ca^{2+}]_i$ in gonadotrophs, while ADP was effective only at submillimolar concentrations. None of these compounds caused permeabilization of the cells. On the other hand, α,β-methylene-ATP, β,γ-methylene-ATP, and UTP had no effect on $[Ca^{2+}]_i$. This pharmacological profile is consistent with the expression of P_2X_2 and/or P_2X_5 purinergic receptor-channels in gonadotrophs. In accord with the physiological role of these channels, ATP was found to stimulate gonadotropin secretion and to enhance agonist-induced gonadotropin release. These observations suggest that ATP can act as a paracrine/autocrine factor in the regulation of Ca^{2+} signaling and secretion in pituitary cells, and that its actions are mediated by P_2 receptor-channels.

Integration of P_2 Channels in Plasma Membrane- and ER-Derived Ca^{2+} Oscillations. Electrophysiological characterization of P_{2X} calcium channels, as well as these channels' integration into the generation of plasma membrane and ER-derived calcium oscillations, was performed in single gonadotrophs. The ATP-induced $[Ca^{2+}]_i$ response was partially inhibited by nifedipine, a blocker of voltage-sensitive calcium channels, but was not by tetrodotoxin-induced blockade of voltage-sensitive Na^+ channels. On the other hand, patch-clamp experiments showed that ATP induced an inward depolarizing current in cells clamped at -90 mV, which is associated with an increase in $[Ca^{2+}]_i$. Thus, the P_2-depolarizing current itself not only drives Ca^{2+} into the cell, but also activates Ca^{2+} entry through voltage-sensitive calcium channels. In accord with this, low [ATP]-induced plasma membrane-dependent $[Ca^{2+}]_i$ oscillations in quiescent cells, and increased the spiking frequency in spontaneously active cells. ATP-induced Ca^{2+} influx also affected agonist-induced and $InsP_3$-dependent $[Ca^{2+}]_i$ oscillations by increasing the frequency, baseline, and duration of Ca^{2+} spiking. These observations indicate that ATP modulates both the plasma membrane and ER oscillators. The effects of ATP on agonist-induced calcium spiking are consistent with the bidirectional effects of calcium on $InsP_3$-controlled calcium release.

Secretion and Degradation of ATP in Pituitary Cells. To further clarify the role of ATP as a positive feedback element in agonist-induced calcium signaling and gonadotropin release, an analysis of ATP secretion and degradation was performed in a mixed population of pituitary cells. Under conditions normally used in secretory studies, no measurable ATP was released from unstimulated and GnRH-stimulated cells. Further experiments revealed that the detection of ATP released from GnRH-stimulated cells was prevented by its rapid degradation by ecto-ATPases. To permit the measurement of ATP secretion, several ecto-ATPase inhibitors were employed, including N-ethyl maleimide, trifluoroperazine, and etharinic acid, but only a minor reduction of ecto-ATPase activity was observed. However, the presence of Ca^{2+}/Mg^{2+} chelators, EGTA or EDTA, in the medium significantly inhibited the degradation of externally added ATP, and GnRH-induced ATP co-secretion with LH readily was readily detectable. In both calcium-containing and calcium-deficient media, only a partial recovery of ATP was observed after employing myokinase/pyruvate kinase enzymatic reactions to which convert AMP and ADP to ATP. In contrast, in perifused pituitary cells exposed to α,β-MeS-ADP, a specific inhibitor of 5'-nucleotidase, the myokinase/pyruvate kinase reactions recovered almost all applied ATP. These observations are consistent with the conclusion that pituitary cells express functional ectoATPase, ecto-ADPase, and ecto-5'nucleotidase, which rapidly degrade ATP in a sequential manner to adenosine, leading to the prompt termination of ATP action as an amplifier of agonist-induced calcium signaling and secretion.

HORMONAL CONTROL OF GONADAL FUNCTION

The Section on Molecular Endocrinology, under the direction of **Maria Dufau**, investigates the molecular basis of peptide hormone action, with particular emphasis on the control of gonadal function. This includes analysis of the structure and function of gonadotropin receptors, as well as the hormone-regulated membrane coupling and intracellular events involved in the modulation of steroid biosynthesis in the testis and ovary. Current research also includes studies on cell-to-cell communication in the testis, developmental aspects of Leydig cell maturation, and induction of regulatory mechanisms in the Leydig cell. This group also investigates the properties and biological activity of stored and circulating gonadotropins in physiologic regulation and in clinical disorders of pituitary and gonadal function.

Role of Cysteine Residues in Hormone Binding and Surface Expression of the LH Receptor. The luteinizing hormone receptor (LHR) is a seven-transmembrane G-protein coupled receptor with a high affinity hormone binding site in the N-terminal extracellular domain. This hormone binding domain is composed of two Cys-rich regions in exons 1 and 9, generally conserved in the glycoprotein hormone receptor family and bordering a leucine-rich domain (exons 2-8). A third Cys-rich region (Cys-314, -321, and -331) is located in the region of the extracellular domain, adjacent to the transmembrane module (exon 10) and well outside the binding domain. This region is also conserved in the FSHR and TSHR. Only Cys-109, Cys-134 (exons 5 and 6), and Cys-282 are not conserved in the FSHR and TSHR. Extracellular loops 1 and 2 (EL1, EL2) contain two Cys residues that are conserved in most GPCRs, and are known to be of structural importance in the adrenergic/rhodopsin receptor family.

To analyze the roles of cysteines and disulfide bonding in LHR hormone binding, all extracellular cysteines including those in EL1 and EL2 of the holoreceptor (Form A), and the corresponding cysteines of the LHR splice variant form B (that includes exons 1-10 containing extracellular domain and a unique 22 amino acid tail with an additional Cys), were replaced by serine residues. The mutant receptors were expressed in COS1 cells or overexpressed in insect cells. The Ser for Cys substitution prevents formation of a putative disulfide bond without significantly affecting the charge characteristics of the protein. The additional Cys-305 in receptor form B cancels potential unpaired cysteines and reduces intermolecular aggregation that could result from reactivity of an unpaired cysteine. These studies demonstrated that only the four non-conserved Cys residues in Exon 1 (Cys-8,-12,-14,-22) are essential, since their individual replacement abolished hormone binding activity. In addition, the two unique Cys residues at 109 and 134 are important since their mutation caused 75% decreases in both cell-surface and total cellular LHR as measured by hormone binding. Each of the four non-conserved Cys residues in exon 1 is essential for hormone binding activity, and no other LHR cysteines can replace their function in the mutant receptors. In studies reported by others, mutation of the analogous Cys residues in exon 1 of the TSH receptor did not affect TSH binding, suggesting that these have a unique function in LH/hCG binding. Since disulfide bonds between Cys residues in exon 1 and Cys- 109 or Cys-134 should yield equivalent loss of binding activity upon mutation of each half-cystine, a disulfide bond may exist between Cys-109 and Cys-134 in the hormone binding domain. The Ser-134 mutant LHR exhibited a marked reduction in maximum agonist-induced cAMP production, consistent with the 75% decrease in binding activity. Substitution of Cys-109 similarly reduced maximal hormonal stimulation. These single substitution experiments demonstrate that specific cysteines in exons 1-6 of the LHR, probably through disulfide bond formation, are necessary for the active configuration of the receptor.

In a related study, another class of cysteines (including extracellular Cys-257,-258,-321, and -331, and EL1-417 and EL2-492) that are not involved in hormone binding were found to be essential for surface expression of the LHR. These mutations resulted in impaired (Cys-282) or total loss of surface expression (all others) in COS-1 cells transfected with the mutant receptors. However, the binding activity of these mutants was recovered in detergent-solubilized extracts of the transfected cells. Other Cys residues, including Cys-282 (exon 10) and Cys-314 (exon 11), were not essential for hormone binding or plasma membrane insertion. Also, the substitution of function by one of the contiguous Cys-257 and -258 residues in exon 9 was ruled out by double mutation of these residues. In addition, radioimmunoassay of receptor protein revealed that although substitution of many of the LHR cysteines caused retention of the mutant proteins in an intracellular membrane fraction, no changes in steady-state concentrations of the receptor were observed. The presence of the correct disulfide configuration necessary to initiate signal transfer may be a prerequisite for membrane insertion, but acquisition of the high-affinity binding conformation is not.

The importance of disulfide bonds in hormone binding activity was investigated in ligand blots using the form B spliced variant overexpressed in insect cells and the affinity-purified Form A holoreceptor isolated from ovarian membranes. Reduction and boiling of the 38 kDa form B receptor prior to electrophoretic separation and transfer to nitrocellulose did not reduce the ability of the electroblotted receptor to bind labeled hCG. These findings demonstrate that the initial oxidation state does not affect the ability of the Form B LHR to renature to the active binding conformation during transfer to nitrocellulose membranes. They also indicate that renaturation of the isolated form B binding domain involves the reformation of exon 1 disulfide bonds, since the primary impact of the conservative substitution of Ser for Cys is the loss of ability to form disulfide bonds. In contrast, oxidation and denaturation of the full length (form A) receptor severely impaired its ability to bind hormone after electrophoresis and ligand blotting. Thus, the presence of the transmembrane/cytoplasmic domains presumably has a negative effect on renaturation to the active binding conformation of the reduced LHR on nitrocellulose. In the form A holoreceptor, reactive cysteines from transmembrane or cytoplasmic domain, exposed only under reducing conditions, may interfere with these putative disulfide interactions in the extracellular hormone binding domain thus and impair ligand binding.

In previous studies, enzymatic digestion showed that folding of the nascent Form B receptor (which lacks the transmembrane/cytoplasmic domain) to an active configuration is dependent on the presence of N-linked carbohydrates at Asn-152 and Asn-173, specifically the N-acetylgalactosamine of the chain attached to asparagine. More recent studies have established that the LHR form B receptor is only capable of spontaneous renaturation during ligand blotting, following SDS denaturation and boiling in the presence or absence of reductant, if the receptor bears the proximal N-acetylglucosamine on Asn-152 and Asn-173, and the four cysteines in exon 1.

Structural Characterization of the Rat Corticotropin Releasing Factor Receptor Gene. The recent identification of several variants of the CRF receptors that carry insertions and deletions of both the extracellular and seven-transmembrane module suggests the presence of multiple introns in the CRF receptor gene, and the expression of splice variants that potentially differ in ligand specificity and modes of signal transduction. These findings by others were relevant to the observation that CRF receptors expressed in the rat Leydig cell differ from the pituitary receptor in not stimulating cAMP production, and does not appear to be coupled to G_s. Activation of this receptor leads through a non-cAMP mediated mechanism to inhibition of the stimulatory action of LH on adenylate cyclase and cAMP production. Such unconventional coupling of the CRFR could reflect a tissue-specific post-translational modification of the receptor, coupling to different G proteins, or the presence of a splice variant of the CRF receptor in the Leydig cell. The structural organization of the CRF receptor gene was determined to permit the elucidation of isoforms and to clarify the signaling potential of the Leydig cell receptor.

The rat CRF receptor gene was isolated from a rat liver genomic library probed with cDNA for the rat pituitary CRFR. The CRFR gene was found to contain 13 exons and 12 introns, and the positions of the exon/intron junctions were similar to those of other Class II G protein-coupled receptor genes including the parathyroid hormone and glucagon receptors. Exons 1-4 correspond to the extracellular domain, exons 5-12 to the transmembrane domain, and exon 13 to the cytoplasmic region. The TATA-less CRFR promoter resides within 593 base pairs of the initiation codon and the major transcriptional start site at nucleotide -238, approximately 20 bp downstream of an AP2 site and 60 bp from an SP-1 consensus element. The addition of the domain between -593 and -651 increased promoter activity in two cell types, indicating that an enhancer or a second promoter is located in this region. Intron junctions were identified in the extracellular, transmembrane, and cytoplasmic domains of the CRFR, giving the potential for differential signal transduction by splice variants of the receptor.

The reported structural variants of the CRF receptor gene were found to correspond to the deletion of complete exons. Exon 3 of the extracellular domain is deleted in-frame from a human receptor variant 3, and does not result in a premature termination codon. A second human variant, the CRF Type 2 receptor, contains an 87-nucleotide insertion after exon 5 of the rat CRF receptor gene. This insertion was not contained within the rat intron between exon 5 and 6, indicating that this specific human variant is not found in the rat. The Leydig cell CRFR mRNAs were expressed at very low levels in comparison with the pituitary, and the predominant form was identified as the rat pituitary CRF receptor sequence form A. Two splice variants (B and C) were also expressed in rat Leydig cells at very low levels. The form B variant recovered by PCR contains a deletion of exon 3 that was previously observed in the human 3 receptor isoform. The positions of the initiation and termination codons in CRFR form B (Leydig cell) have not yet been identified, but the signal sequence of this clone (exon 1) differs from that of the Form A receptor. A second rat Leydig cell variant (form C) was amplified with a 5' primer begining in exon 3 at position 215 and continues to exon 13 with deletions of exons 7, 11, and 12. Exons 1 or 2 were not amplified with form A primers, indicating a novel amino terminal sequence. The PCR product begins with amino acid 73 in exon 3. Deletion of exon 7 results in a frameshift with premature termination after a unique 39 amino acid tail that contains two hydrophobic transmembrane domains. It remains to be determined whether this form of the CRFR is responsible for the unique properties of the Leydig cell receptor. Because the pituitary form is predominant among the low-abundance CRFR variants expressed in the Leydig cell, it is conceivable that the lack of coupling to G_s is related to receptor density. However, it is also possible that the relevant variant involved in CRF-induced inhibition of gonadotropin action in Leydig cells has yet to be identified.

The CRF receptor gene exhibits substantial similarity to the glucagon/PTH receptor gene family, and is characterized by the presence of introns within its transmembrane/cytoplasmic module, highly conserved cysteines in its extracellular domain, and a highly conserved first intracellular loop. The CRF receptor exon/intron junctions align with those of the PTH and glucagon receptors after exons 3,5,7,8,9,10, and 12. Exons 11 and 12 are divided in the CRFR and PTHR, but are contiguous in the glucagon receptor. All members of this receptor family mediate both cAMP generation and phosphoinositide hydrolysis, consistent with the similarity of their transmembrane domains and intracellular loops.

The positions and sequences of the cleavage sites of the introns of the seven transmembrane (TM) module of the CRFR/PTHR family seem to be related to the positions of alternative splice sites in the intronless 7TM module of the LHR that produce the splice variants. An alternative acceptor splice site at position 1895 within the intronless LHR exon 11 module that yields a splice variant, initially identified in the pig as Form D, is positioned at the same location as intron 12 of the CRFR, immediately following TMVII. Thus, the alternative splice sites in the LHR coding region substitute for the 3' LHR intronic splice site and serve as a cleavage site for the spliceasome complex, and possibly as a site of intron insertion later in evolution. This observation is also consistent with the fact that the 3' element of CRFR exon 11, which is a cleavage site in the CRFR and PTHR, but not in the GluR, conforms to the LHR TTXCAG 3' intron J splice element, and the alternative splice sites that produce LHR forms B and C. CRF exons 1,4,5,10,11, and 12 terminate in the 3' intron acceptor consensus element AG, indicating that these positions may have served as alternate acceptor cleavage sites in an intronless precursor, and later as intron insertion points. Based on these studies, it is proposed that a mechanism of CRF Class 2 intron insertion in evolution may have been derived from the intronless exon 11 of the LHR.

Multiple and Tissue-specific Control of Prolactin Receptor Gene Expression. The group has recently succeeded in cloning the rat prolactin receptor gene, and have identified tissue-specific promoters that control the expression of the receptor in specific target tissues including the liver, gonads, and mammary gland. Expression of the rat prolactin receptor gene is controlled by at least three promoters, PI, PII, and PIII, which precede non-coding exons $E1_1$, $E1_2$ and $E1_3$, respectively. These are alternatively spliced to a common non-coding exon that precedes the exon containing the translation initiation codon. Three alternative first exons are utilized in a tissue-specific manner: $E1_1$ is predominantly present in the ovary, $E1_2$ is specific for the liver, and $E1_3$ is transcribed from the common promoter PIII and is the major form in Leydig cells; it is the minor form in the ovary and the liver. PIII was found to direct transcription of the PRLR gene in the rat mammary gland, where PI and PII were not detected. Such multiple and tissue-specific promoter utilization may provide a molecular basis for the differential regulation of PRLR expression in normal as well as neoplastic tissues. Analysis of the orientation of the three alternative first exons by mapping several overlapping genomic clones revealed that PIII is localized upstream of PI and PII. The use of genomic FISH (fluorescence *in situ* hybridization) revealed that the the three alternate first exons are colocalized to the same chromosomal locus, as is the PRLR coding region, indicating that the rat PRLR gene is transcribed alternatively from at least three promoter regions. Alternate utilization of exons accounts for the multiple forms of 5' UTRs in the PRLR transcripts. Promoter deletion studies performed in mouse Leydig tumor cells (MLTC) and HepG2 cells have localized the minimal promoter domains of PI, PII, and PIII to regions -700 to -549 (5', flanking the initiation site at -549), -606 to -179 (encompassing the initiation site at -405) and -607 to -181 (encompassing the initiation site at -340), respectively. PI exerts strong promoter activity only in MLTC, PII exerts strong activity only in HepG2, and PIII is operative in both cells types. In the rat, the discrepancy noted between the tissue mRNA transcripts related to PI *in vivo,* and the promoter activity of PI constructs in cultured Leydig cells, could be due to species differences (PI is exclusively active in ovarian tissue in the rat and is not present in the testis). Suppression of PI probably does not occur in MLTC-1 tumor cells, but is observed in rat testicular Leydig cells *in vivo*. At present there are no available homologous rat Leydig or ovarian cell lines for further studies of tissue-specific differences observed *in vitro* and *in vivo*. Subsequent studies will be performed using primary cultures of Leydig and ovarian follicular cells.

The Androgen Pathway: Hormonal Regulation of Testicular Function. Studies of the regulation of androgen synthesis in steroidogenic cells have focused on both transcriptional and post-transcriptional regulation of the proteins that catalyze these reactions; the P450 17α that catalyzes the production of DHEA or androstenedione by consecutive hydroxylase and lyase reactions, and the 17β-hydroxysteroid dehydrogenase (17β-HSD) that catalyzes the conversion of androstenedione to testosterone. Studies on the regulation of CYP17 lyase activity at the molecular level have utilized species and tissue specific differences to identify target regulatory sequences. In primary cultures of rat adrenal and rat Leydig cells, adenovirus infection of CYP17 promoter/luciferase reporter gene constructs revealed a rat-specific domain between -1 and -180 bp that caused lack of both basal and cAMP-induced CYP17 transcription in the adrenal, but not the Leydig cell. In contrast, similar promoter constructs from other species (human and bovine) exhibited substantial cAMP-induced transcriptional activity in the rat adrenal. Functional studies indicate that the presence of the rat CRE/ATF domain, or the absence of the bovine/human SF-1 domain, at this aligned position, is not responsible for silencing rat gene transcription in the rat adrenal. This domain includes an additional SF-1-like element that overlaps with the rat SF-1 element in these constructs. These studies suggest that the differential CYP17 activity of rat Leydig and adrenal cells may depend on a transcription factor that binds to the rat gene but not the human gene, and is specific to only one of the two cell types, Leydig or adrenal. Whereas the rat and human promoters are driven by an SF-1 protein, which is present in both steroidogenic cell types, a non-SF-1 inhibitor (adrenal) or activator (Leydig) trans-factor may be responsible for the tissue-specific promoter activity.

Structural studies based on mutagenesis of the conserved region of the rat and human enzymes revealed significant differences in the amino acid domains required for hydroxylase and lyase activities within and between species (human/bovine vs rat), consistent with their differential regulation of lyase activity. The conserved 334 to 363 domain contains a sequence that is highly similar to that of the signature sequence in the active site of the short-chain dehydrogenase family (YGXSK vs. YVGXSR339). Furthermore, a prokaryotic member of that family (3α/20β HSD) also contains significant similarity to the essential arginines in this domain. Mutagenesis of R339 from the rat CYP17 (and also R340 of the human CYP17) signature sequence abolished both hydroxylase and lyase activities without affecting protein expression levels. These findings indicate the crucial importance of this residue for CYP17 activity, and suggesting a structural link between the CYP17 active site and the short chain dehydrogenase steroid binding domain.

Studies on the mechanism of differential inhibition of lyase activity in the human enzyme utilized rat P450 17α-hydroxylase, which catalyzes both Δ_4 and Δ_5 lyase activities (the human enzyme only catalyzes Δ_5 lyase activity). The previous observation that Arg346 is essential only for rat CYP17 lyase activity suggested that this amino acid is either an essential contact point for the 17α-hydroxylated steroid, or is necessary for selective stimulation of lyase activity by tissue-specific factors. Sequential mutations of the amino acids surrounding Arg 346 in the rat protein suggested that F343 contributes specifically to Δ_4 lyase activity. Substitution by the human counterpart, isoleucine, substantially reduced Δ_4 lyase activity, whereas hydroxylase activity was not changed. To determine whether the F343 to I344 substitution in the human CYP17 is responsible for its lack of Δ_4 lyase activity, the human I344 residue was mutated to the rat F residue. The mutant human P450 17α-hydroxylase exhibited about 50% of the rat Δ_4 lyase activity, indicating that I344 inhibits lyase activity but is not completely responsible for this species difference. However, this mutation also resulted in a change in hydroxylase activity in the human protein. Further mutations of amino acids in this domain to the rat counterparts confirmed that the 344/349 sequence is important for hydroxylase activity in the human protein, and that the corresponding rat sequence cannot substitute for this. These observations have identified amino acids in the 343/363 domain of the rat and human enzymes that are selectively important for hydroxylase or lyase activities to varying degrees. It is likely that this domain serves as a steroid binding domain, rather than as a general surface conduit for activators.

ROLE OF PROTEIN PHOSPHORYLATION AND OXIDATION IN CELL REGULATION

The Section on Metabolic Regulation, directed by **Kuo-Ping Huang**, studies the role of protein phosphorylation and oxidation in cellular regulation. Covalent modifications of proteins by phosphorylation and oxidation play important roles in mediating many cellular events resulting from ligand-receptor interactions that generate a variety of second messengers, including cAMP, cGMP, 1,2-diacylglycerol, arachidonic acid, inositol 1,4,5-trisphosphate, Ca^{2+}, and nitric oxide. An increase in $[Ca^{2+}]i$, either through ion channels or triggered by inositol 1,4,5-trisphosphate released from membrane phospholipids, stimulates the protein kinase C enzyme family when 1,2-dicylglycerol and free fatty acids levels are also increased. Phosphorylation of target substrates by protein kinase C has been linked to the regulation of cell growth and differentiation, secretion, cell surface receptor function, cellular metabolism, and learning and memory. In the CNS, the excitatory amino acid-mediated opening of receptor ion channels and the resulting increase in $[Ca^{2+}]_i$ also stimulates nitric oxide synthase. One of the targets of nitric oxide is the sulfhydryl group of proteins. Modification through nitrosylation, as well as disulfide bond formation, causes conformational changes of proteins and influences their biological activities.

Oxidation of Rat Brain Neurogranin by Nitric Oxide. Neurogranin (Ng) is a 78-amino acid postsynaptic neuronal protein that binds calmodulin (CaM) with high affinity at low Ca^{2+} levels. Ng is phosphorylated by protein kinase C (PKC) at a single site within a conserved 19-amino acid region that has homology with neuromodulin and contains the predicted CaM-binding domain. Phosphorylation by PKC reduces the binding affinity of Ng and neuromodulin for CaM, and has been proposed to free CaM for activation of other CaM-dependent enzymes. Rat brain Ng, which contains four cysteine residues, was found to be readily oxidized by nitric oxide donors such as DEANO and sodium nitroprusside. This leads a large conformational change that is detectable as a marked decrease in apparent size (from 17 to 10 kDa) on non-reducing SDS-polyacrylamide gel electrophoresis, and is reversed by reduction with sulfhydryl reagent. Both non-phosphorylated and PKC-phosphorylated Ng were susceptible to the oxidation-induced change in conformation, which was prevented by chemical modification of all four cysteine residues. The oxidative modification of Ng by DEANO was inhibited by CaM in the absence but not in the presence of Ca^{2+}. CaM had no protective effect on DEANO-mediated oxidation of the PKC-phosphorylated Ng with or without Ca^{2+}. Oxidation of Ng causes intramolecular disulfide bond formation that accounts for its smaller molecular size on SDS-gel electrophoresis. The oxidized form of Ng displayed lower affinity for CaM than the reduced form as evidenced by CaM-affinity chromatography. Ng ranked as one of the best nitric oxide acceptors among those tested, including dithiothreitol, glutathione, and serum albumin. The Ng associated with rat brain synaptosomes is also oxidized by DEANO as determined by immunobloting with a specific antibody. Ng in rat brain extracts is readily oxidized in the presence of Ca^{2+} and this is reversed by NADPH, suggesting the existence of enzymes that catalyze the interconversion of Ng between its oxidized and reduced states. These findings suggest that Ng is a physiological target of nitric oxide as well as PKC, and that modification of Ng by nitric oxide and other oxidants could serve as a signaling mechanism for controlling the availability of CaM, which is essential for many Ca^{2+}-regulated cellular processes.

Site-directed Mutagenesis of Rat Brain Neurogranin to Identify Cysteine Residues Involved in Intramolecular Disulfide Bond Formation. Rat brain Ng contains four cysteine residues, at positions 3,4,9 and 51, all of which are potential targets of nitric oxide. Modification of Ng by nitric oxide is probably mediated by the production of a nitroso intermediate followed by disulfide bond formation. Ng constructs containing single, double, and quadruple mutants of the four cysteine residues were generated for examining the role of individual cysteines in forming intramolecular disulfide bonds. Analysis of the mutant proteins produced in *E. coli* revealed that mutation of all four Cys residues, or a single mutation of Cys^{51}, prevented the oxidant-induced formation of intramolecular disulfide bonds as monitored by the mobility shift on SDS-PAGE. Single mutations of Cys^{3}, Cys^{4}, or Cys^{9}, or double mutations of any pair of these three Cys residues, altered the rates of oxidation of the mutant proteins but did not block intramolecular disulfide formation. Thus, Cys^{51} is an essential pairing partner in NO-mediated intramolecular disulfide bond formation in Ng. Cys^{3}, Cys^{4}, and Cys^{9} individually could pair with Cys^{51} with an order of reactivity of $Cys^{9} > Cys^{4} > Cys^{3}$, suggesting that Cys^{9} and Cys^{51} form the preferential disulfide bridge. The intramolecularly disulfide-bridged Ng proteins had markedly attenuated CaM-binding affinity and 2 to 3-fold lower PKC substrate phosphorylation activity. These findings indicate that the N-terminal Cys^{3}, Cys^{4}, and Cys^{9} are in close proximity to the C-terminal Cys^{51} in solution. Disulfide bridging between the N- and C-terminal domains of Ng renders the conformation of the central binding/phosphorylation domain unfavorable for CaM binding and phosphorylation by PKC. The Cys^{51} protein mutant is similar to bovine Ng and a human Ng-2 variant, which do not appear to undergo the intramolecular disulfide bonding that occurs in rat Ng.

Cloning and Properties of Bovine, Chicken, and Human Neurogranin cDNAs. To determine whether Cys^{9} and Cys^{51} are crucial for nitric oxide-mediated intramolecular disulfide bond formation in other species, the properties of bovine, chicken, and human Ng were investigated. PCR cloning of bovine Ng cDNA revealed a single amino acid substitution of the rat Ng Cys^{51} with Arg^{51}. Consistent with this finding, bovine Ng expressed in *E coli* did not form an intramolecular disulfide bond after oxidation but instead formed polymers as a result of intermolecular disulfide bond formation. Furthermore, in cloned chicken Ng the Cys^9 and Cys^{51} residues are replaced by Ser^9 and Gly^{51}, respectively, and the protein is predominantly monomeric. Of two human Ng cDNAs that were cloned from human brain mRNA, Ng-1 contains Gly^{51} and Ser^9 and Ng-2 contains Arg^{51} and Cys^9; the former is similar to chicken Ng and the latter to bovine Ng. The expressed human Ng-1 and Ng-2 proteins behaved like the chicken and bovine Ng, respectively, upon oxidation. Comparison of the nucleotide and amino acid sequences of rat, bovine, chicken, and the two human clones indicates that variations among the Ngs occur at positions 9 and 51. Single point mutations of rat Ng codon 9 from TGC to AGC converts Cys to Ser for both chicken and human Ng-1. Single point mutation of rat codon 51 from TGT to CGT converts Cys to Arg for bovine Ng; mutation to GGT converts Cys to Gly for both chicken and human Ng-1. Single point mutation of bovine Ng codon 51 from CGT to CGC for human Ng-2 retains Arg^{51} for both clones. The PKC phosphorylation site and the CaM-binding domain of all the Ng clones are completely conserved among species. These findings suggest that the neurogranins of different species are similarly regulated by PKC, but differ in their responses to oxidant-mediated signaling.

Characterization of Promoter Activity in the 5' Flanking Region of the HASPP28 Gene. HASPP28 is a ubiquitous phosphoprotein that can be phosphorylated by several kinases including PKA, PKC, and CK1 and 2. However, in intact cells its phosphorylation seems to be catalyzed by a CK2-like kinase, and its expression and phosphorylation appear to be regulated during the cell cycle. To better understand the regulation of its expression and phosphorylation, the HASPP28 gene was cloned and its 5'-flanking region was characterized. Two genomic fragments (+509 to -189 and +82 to -1231) were identified by PCR cloning. The first fragment covered more than 90% of the coding region without interruption by introns. Primer extension experiments identified multiple transcription start sites, the most distal located -55 nt from the translation start site. Computer analysis of the entire 5' flanking sequence of revealed numerous putative transcription regulation elements. Three TATA boxes and three TFIID element sequences were present, including one of each within 150 bp proximal to the transcription initiation site. Many transcription regulatory elements within the 1.2 kb 5' flanking region were paired, some in the same orientation and the majority in the antiparallel orientation, suggesting dimerization of the transactivation binding proteins. A series of deletion constructs upstream from the translation start site were fused with a luciferase-carrying pGL2 reporter plasmid and transfected into COS-7 cells to characterize the promoter activities of the 5' flanking sequences. The results indicated that the basal elements required for transcription are located -582 nt to -382 nt upstream from the translation start site. The construct with the longest sequence did not exhibit the strongest promoter activity, suggesting the presence of a suppressor element between -1231 and -1021. The several constructs exhibited only 2-5% of the activity of the positive control plasmid pGL2-SV40 promoter, implying that a potent activating element is situated upstream of -1231 nt or that the element(s) within this sequence are highly responsive to extracellular stimuli that are not operative under the present assay conditions. Of several potential modulators tested for their effects on promoter activity in COS cells, EGF, dBu-cAMP, and PMA caused 2-3 fold increases in the luciferase activity, while PDGF (AA or BB), bFGF, and insulin had little effect. A unique ATF element for CREB (tgacgat) is situated at -1101 nt and a unique TRE/AP1 site (gtgactca) at -170 nt, and account for the stimulatory actions of dBu-cAMP and PMA.

Antisense Regulation by PKC of Neuritic Outgrowth from Neuroblastoma Cells. Neurite outgrowth (NOG) in N1E115 neuroblastoma cells is induced by dBu-cAMP and inhibited by phorbol esters such as PMA. In these cells, PMA treatment rapidly translocates and down-regulates PKC α and δ, slowly translocates and only slightly down-regulates PKC β1, and leaves PKC ξ unaffected. After prolonged exposure to PMA, when PKC α and δ are completely down-regulated, further PMA treatment no longer inhibits dBu-cAMP-mediated NOG, suggesting that either PKC α or δ (or both) are responsible for the inhibition of cAMP-mediated NOG. To identify the roles of the individual

PKC enzymes, stable transfectants were developed in which either PKC α or δ levels were reduced by transfection with a pCDNA3 vector containing antisense DNA to the 5' untranslated region of PKC α or δ, respectively. Several NIE 115/ASα and /ASδ clones were established, and showed various degrees of reduction in PKC α and δ levels as analyzed by immunoblotting. Clones with stably elevated PKC α expression through transfection with the full length sense sequence were also established. Morphological examination indicated that representative clones of ASα and ASδ showed higher sensitivities to dBu-cAMP in terms of NOG in the presence or absence of PMA, while elevated PKC α rendered these cells more resistant to the action of cAMP. As before, PMA no longer inhibed cAMP-mediated NOG after PMA pretreatment. Although PMA normally retracts the neurites elaborated during cAMP treatment, this effect was diminished in cells with reduced levels of PKC α or δ. These observations demonstrate that both PKC α and δ are probably involved in the inhibition of cAMP-mediated NOG in N1E115 neuroblastoma cells.

ADRENAL STEROIDOGENESIS

The Section on Steroid Regulation, led by **Charles Strott**, investigates the processes and mechanisms involved in the biotransformation of steroids by conjugation to highly charged chemical groups such as sulfonate. The sulfonation of biomolecules occurs widely across species and causes dramatic changes in the physicochemical properties of the sulfonated compounds. Thus, the sulfo-conjugation of steroids results in the conversion of these essentially hydrophobic compounds to a more water soluble state. While the biological importance of steroid sulfonation is poorly understood, it has several possible functions. For example, by increasing steroid polarity and thereby altering water solubility and protein binding, sulfonation can influence steroid transportability. It may also function as an initiating or termination event to produce active or inactive molecular species, and to regulate physiological responsivity to steroids, Sulfonation, the transfer of a sulfonate group (SO_3^-) from a universal donor to an appropriate acceptor site, is catalyzed by enzymes termed sulfotransferases. One of the most active tissues in steroid sulfonation is the adrenal cortex, where members of the steroid sulfotransferase family are differentially expressed by functionally distinct cells. Current efforts to shed light on the biological consequences of steroid sulfonation include the isolation, cloning, and expression of adrenocortical steroid sulfotransferases, as well as investigation of the enzyme systems responsible for the synthesis of the universal sulfonate donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS).

The Steroid Sulfotransferase Active Site. Steroid sulfotransferases interact with two substrates, the sulfonate donor and the steroid acceptor molecules. A GxxGxxK sequence, related to the P-loop motif commonly found in ATP- and GTP-binding proteins, is positioned within a highly conserved region located near the C-termini of all steroid and phenol sulfotransferases for which primary structures are known. To analyze the role of the GxxGxxK motif in the binding of PAPS, point mutations that replaced the conserved glycines (G260 and G263) and lysine (K266) with alanines were introduced singly or in combination into guinea pig estrogen sulfotransferase (gpEST) cDNA. As controls, non-conserved residues (S262, D264, and N267) within the motif were mutated to alanine either singly or in combination. Nine mutant constructs generated from wild type gpEST cDNA were all found to express the 34 kDa gpEST protein as verified by immunoblot analysis. The expressed proteins were subjected to kinetic analysis to detect perturbations in their interaction with both PAPS and the estrogen substrate. While the mutation of a single motif residue had little effect on the interaction between gpEST and PAPS as determined by kinetic analysis and photoaffinity labeling, simultaneous mutation of any two residues caused a 10-fold increase in the K_m for PAPS and reduced photoaffinity labeling. Mutation of all three motif residues resulted in an inactive enzyme and complete loss of photoaffinity labeling, although the protein was expressed in reasonable amount. On the other hand, the K_m for PAPS was unchanged in the "control" mutants, demonstrating the specific importance of the conserved motif residues. Several mutant constructs also displayed a striking change in K_m for the estrogen substrate, with greater perturbations (8- to 28-fold increase in K_m) for double than for single mutations. Whereas mutations of non-conserved motif residues had a negligible effect on the K_m for PAPS, there was a marked increase in the K_m for the estrogen substrate (>30-fold). These data indicate that G260, G263, and K266, amino acid residues that constitute the GxxGxxK P-loop motif, play a critical role in the interaction between estrogen sulfotransferase and the universal sulfonate donor, PAPS. In addition, the specific G260 and N267 residues are essential for efficient sulfonation of the estrogen substrate. These findings indicate that the sequence GISGDWKN, encoded by the 5' end of the 3' terminal exon of the gpEST gene, represents an essential component of the active site for gpEST. Also, since the GxxGxxK motif is universally conserved, this area should be catalytically important for all steroid and phenol sulfotransferases.

Molecular Cloning of a Second 3-Hydroxysteroid Sulfotransferase. A second guinea pig adrenal 3-hydroxysteroid sulfotransferase (gpHST2) cDNA has been cloned that is distinct from the previously cloned chiral-specific 3α-hydroxysteroid sulfotransferase (gp3αHST1, now designated gpHST1). The deduced amino acid sequences for gpHST1 and gpHST2 are 86% identical; however, whereas gpHST1 acts stereoselectively on 3α-hydroxylated neutral steroids, gpHST2 demonstrates a clear preference (but not exclusive specificity) for 3β-hydroxylated steroids. Thus, gpHST2 is similar to a previously isolated gpHST that selectively acted on 3β-hydroxylated steroids (gp3βHST). In addition, gpHST2 at 33 kDa is the same size as gp3βHST and larger than gpHST1 (32 kDa). gpHST2 also contains amino acid sequences identical to peptides obtained from gp3βHST and cross-reacts with antibodies raised against purified gp3βHST. Nonetheless, gpHST2 can sulfonate both 3α- and 3β-hydroxylated neutral steroids, suggesting that either gp3βHST does not have the exquisite stereoselectivity previously indicated, or this subfamily of hydroxysteroid sulfotransferases is larger than originally recognized.

Isolation and Characterization of Guinea Pig PAPS Synthetase. The activation of inorganic sulfate to form PAPS results from the concerted actions of ATP sulfurylase (SUL) (reaction #1) and APS kinase (KIN) (#3):

1) $\mathrm{ATP} + \mathrm{SO_4^{2-}} \rightarrow \mathrm{APS} + \mathrm{PP_i}$
2) $\mathrm{PP_i} + \mathrm{APS} \rightarrow \mathrm{ATP} + \mathrm{SO_4^{2-}}$
3) $\mathrm{APS} + \mathrm{ATP} \rightarrow \mathrm{PAPS} + \mathrm{ADP}$

Since reaction #1 is thermodynamically unfavorable in the forward direction, the more favorable reverse reaction (#2) is used to assay SUL activity, utilizing [^{35}S]APS and radioinert pyrophosphate (PP_i) to measure the formation of [$^{35}SO_4$]. APS kinase activity is measured by the formation of radioactive PAPS using [^{35}S]APS and ATP (reaction #3). During isolation of SUL and KIN from guinea pig adrenal cytosol, the two activities co-purified throughout purification by ammonium sulfate precipitation, S-200 gel filtration, ion-exchange chromatography, and PAP-affinity chromatography. The active fraction eluted from the affinity gel contained a single protein band of 55 kDa when analyzed by SDS gel electrophoresis and silver staining. Isoelectric focusing of the same preparation revealed two bands with pIs of 6.8 and 6.9 suggesting charge isoforms. The bifunctional activity of the purified 55 kDa protein (PAPS synthetase) is in contrast to the situation in bacteria, fungi, yeast, and plants, where SUL and KIN activities are associated with distinct proteins.

Interestingly, inorganic sulfate was consistently formed even with the most highly purified enzyme preparation. While this raised the possibility of an intrinsic PAPS sulfatase activity, it was initially presumed to result from either contamination with a sulfatase or possibly the backward reaction

(reaction #2) as a result of PPi contaminating the ATP preparation. In regard to the first possibility, it was reasoned that a contaminating sulfatase would utilize exogenous PAPS and form inorganic sulfate in the presence of the same cofactors, metal ions and assay conditions. However, only a minuscule amount of exogenous PAPS hydrolysis was detected, suggesting that no contaminating sulfatase was present in the purified preparation. In regard to the second possibility, treatment of the purest ATP preparation with pyrophosphatase (to degrade any PP_i that might be present) prior to enzymatic analysis did not alter the formation of free sulfate. It was concluded that the inability of the most purified preparation to utilize exogenous PAPS to form sulfate, and the failure of pyrophosphatase to alter the results, indicates that PAPS sulfatase activity might indeed be intrinsic to PAPS synthetase. When KIN was assayed in the presence of 250 μM ATP, the [^{35}S]APS substrate was quantitatively converted to [^{35}S]PAPS with no formation of free [$^{35}SO_4$]. However, when 5 mM ATP was used in the assay, PAPS formation was essentially undetectable and [$^{35}SO_4$] accumulation occurred instead. Thus, the intrinsic PAPS sulfatase activity of the most pure preparation of PAPS synthetase was dependent on the concentration of ATP. No [^{3}H]AMP was formed when [^{3}H]APS was used as the radioactive substrate, eliminating ATP-dependent APS sulfatase activity. Thus, the sulfatase activity intrinsic to purified PAPS synthetase is a PAPS-specific sulfatase that is ATP-dependent. At this juncture, PAPS synthetase appears to be a multifunctional protein responsible for the production of two compounds of importance in sulfotransferase reactions, namely PAPS and PAP(3',5'ADP). The former is essential for sulfonation as the universal sulfonate donor molecule, and the latter has two apparent roles. PAP was previously found to be a potent inhibitor of sulfonation by EST, a reaction that involves a basic isoform of EST; at the same time PAP promotes high-affinity estradiol binding to an acidic isoform of EST. This level of biochemical and physiological complexity is not easily understood in our current state of knowledge about these unusual enzyme systems.

Cloning and Expression of Human PAPS Synthetase. Expressed sequence tags (est) from a directionally cloned human infant brain cDNA library were searched for a clone with sequence homology to yeast APS kinase. Such a clone (est07073) was obtained from ATCC (HIBBQ-19). Since a 480 bp stretch of the 5'-end of HIBBQ-19 had been sequenced, the deduced 160 amino acid sequence was available. The HIBBQ-19 clone was amplified and the plasmid isolated for use as a sequencing template. A sense primer based on the conserved FISP region of APS kinase in E. coli and S. cervisiae was synthesized and used for sequencing. By contiguous cDNA walking using eight overlapping sequence primers, a continuous sequence of 2098 bp was obtained to the poly-A tail. The 5'-end was completed using the 5'-RACE procedure. The full-length human PAPS synthetase (hPAPSS) cDNA consists of 2221 bp. Cytosol prepared from COS-1 cells transfected with the hPAPSS cDNA contained significant activities (2-3 x) for ATP sulfurylase and APS kinase over background compared to nontransfected cells, demonstrating the bifunctionality of the expressed protein.

ATP sulfurylase and APS kinase have been cloned from bacteria, fungi, yeast, and plants, where the enzymes are found on separate polypeptide chains. In these species the Mr of ATP sulfurylase ranges from 35.2 to 63.9 kDa, while the Mr of APS kinase ranges from 22.3 to 29.7 kDa. Interestingly, in contrast to what is found in bacteria, fungi, yeast, and plants, ATP sulfurylase and APS kinase isolated from mammalian tissues, such as rat chondrosarcoma tissue and the guinea pig adrenal (vide supra), are physically linked on a single bifunctional protein (Mr 56 kDa and 55 kDa, respectively). In confirmation of this finding, a cDNA was recently cloned from a marine worm that expresses a 57 kDa protein containing both ATP sulfurylase and APS kinase activities. Subsequently, a mouse cDNA was cloned expressing a correspondingly bifunctional PAPS synthetase protein.

Worm PAPS synthetase has a calculated molecular mass of 68397 daltons and pI of 6.23; mouse PAPS synthetase has a calculated molecular mass of 70713 daltons and a pI of 6.30; and human

PAPS synthetase has a calculated molecular mass of 61621 daltons and a pI of 6.40. Amino acid sequence alignment of the three proteins reveals 362 identities (58%). The worm and human PAPS synthetases are 59% identical (identities increase to 68% when length differences are considered). Remarkably, the mouse and human PAPS synthetases are 86% identical, and this increases to 96% when length differences are taken into account. There is clearly a marked conservation of structure for PAPS synthetase, as illustrated for such widely spaced species as worms, mice, and human beings. An interesting structural feature of the three PAPS synthetase proteins is that each contains a nucleotide-binding motif near their N-termini that is termed a P-loop for ATP- and GTP-binding proteins. The primary structure of a P-loop typically consists of a glycine-rich region followed by a conserved lysine. An identical P-loop motif is conserved for APS kinase in bacteria, yeast, and plants; on the other hand, ATP sulfurylase from bacteria, yeast, fungi, and plants does not contain a similarly conserved motif. It is further of interest that a related P-loop motif is present in all steroid and phenol sulfotransferases cloned to date (vide supra).

The fusion of ATP sulfurylase and APS kinase into a single polypeptide chain could provide an adaptive advantage by facilitating the "channeling" of APS from ATP sulfurylase to APS kinase. This rationale is based on the substantial energy barrier to APS formation (vide supra), and the reported instability of APS in cells. Thus, development of a *fused* protein system could represent an evolutionary step that creates a more efficient pathway to overcome inherent obstacles to the synthesis of activated sulfate and production of this universal sulfonate donor molecule.

Publications:

Arora KK, Sakai A, Catt KJ. Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. J Biol Chem, 1995;270:22820-22826.

Arora KK, Cheng Z, Catt KJ. Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor. Mol Endocrinol 1996, 10:979-986.

Bagnato A, Tecce R, Moretti C, DiCastro V, Spergel D, Catt KJ. Autocrine actions of endothelin-1 as a growth factor in human ovarian carcinoma cells. Clin Cancer Res 1995;1:1059-1066.

Catt KJ. Hormone receptors. In: Hillier SG, Kitchener HC, Neilson JP, eds. Scientific essentials of reproductive medicine. London: WB Saunders, 1996;32-44.

Catt KJ. Intracellular signaling. In: Hillier SG, Kitchener HC, Neilson JP, eds. Scientific essentials of reproductive medicine. London: WB Saunders, 1996;45-59.

Catt KJ, Hunyady L, Ji H, Tian Y, Baukal AJ, Clark AJL, Balla T. Structure-function properties and signaling pathways of the angiotensin AT_1 receptor. In: Vinson GP, Anderson DC, eds. Adrenal glands, Adrenal glands, vascular system, and hypertension. Bristol: Journal of Endocrinology, 1996; 1-19.

Cawley NX, Chen, HC, Beinfeld MC, Loh YP. Specificity and kinetics studies on the cleavage of various prohormone mono- and paired basic residue sites by yeast aspartic proteases 3. J Biol Chem 1996;271:4168-4176.

Cesnjaj M, Zheng L, Catt KJ, Stojilkovic SS. Dependence of stimulus-transcription coupling on phospholipase D in agonist-stimulated pituitary cells. Mol Biol Cell 1995;6:1037-1047.

Chiba H, Komatsu K, Lee YC, Tomizuka T, Strott CA. The 3'-terminal exon of the family of steroid and phenol sulfotransferase genes is spliced at the N-terminal glycine of the universally conserved GxxGxxK motif that forms the sulfonate donor binding site. Proc Natl Acad Sci USA 1995;92:8176-8179.

Downing, GJ, Kim, S, Nakanishi, S, Catt KJ, Balla, T. Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. Biochemistry 1996;35:3587-3594.

Driscoll WJ, Komatsu K, Strott CA. Proposed active site domain in estrogen sulfotransferase as determined by mutational analysis. Proc Natl Acad Sci USA 1995;92:12328-1233.

Dufau, ML. The luteinizing hormone receptor. Current Opinion in Endocrinology and Diabetes. Vol. 2, 2:365-374, 1995.

Dufau ML. The luteinizing hormone receptor. in "The Leydig Cell" (Payne A, Russel L, Hardy M, eds.) Cache River Press, Clearwater, FL, p. 333-350, 1996.

Dufau ML, Miyagawa Y, Takada S, Khanum A, Miyagawa H, Buczko E. Regulation of androgen synthesis: The late steroidogenic pathway. Steroids. (In Press) 1996.

Ehrlich A, Barnett VA, Chen HC, Schoenberg M. The site and stoichiometry of the N-phenylmaleimide reaction with myosin when weakly-binding crossbridges are formed in skinned rabbit psoas fibers. Biochim Biophys Acta 1995; 1232:13-20.

Goodfriend TL, Elliott ME, Catt KJ. Angiotensin receptors and their antagonists. New England J Med 1996;334:1649-1654.

Hong J, Zheng W, Zheng V, Catt KJ, Sandberg K. Genetic transfer of a nonpeptide binding site to a previously unresponsive angiotensin receptor. Proc Natl Acad Sci USA 1995;92:9240-9244.

Hu Z, Zhuang L, Dufau ML. Multiple and tissue-specific control of gonadal and non-gonadal prolactin receptor gene expression. J Biol Chem 271:10242-10246, 1996.
Hunyady L, Balla T, Catt KJ. The ligand binding site of the angiotensin AT_1 receptor. Trends Pharmacol Sci 1996;17:135-140.

Hunyady L, Zhang M, Jagadeesh G, Bor M, Balla T, Catt KJ. Dependence of agonist activation on a conserved apolar residue in the third intracellular loop of the AT_1 angiotensin receptor. Proc Natl Acad Sci USA (in press).

Johnson BE, Damadaran A, Rushin J, Gross A, Le PT, Chen HC, Harris RB. Ectopic production and processing of atrial natriuretic peptide in a small cell lung cancer cell line and tumor from a patient with hyponatremia. Cancer (in press).

Kapas S, Catt KJ, Clark, AJL. Cloning and expression of cDNA encoding a rat adrenomedullin receptor. J Biol Chem 1995;270:25344-25347.

Krsmanovic LZ, Stojilkovic SS, Catt KJ. Pulsatile gonadotropin-releasing hormone release and its regulation. Trends Endocrinol Metab 1995; 7:56-59.

Krsmanovic LZ, Stojilkovic SS, Catt KJ: Pulsatile GnRH release and its regulation. Trends Endocrinol Met 1996; 7:56-59.

Kukuljan M, Vergara L, Stojilkovic SS: Modulation of the kinetics of InsP$_3$-induced [Ca^{2+}]$_i$ oscillations by calcium entry in pituitary gonadotrophs. Biophys J 1996 (in press).

Lee-Huang S, Huang PL, Huang PL, Bourinbaiar AS, Chen HC, Kung SF. Inhibition of human immunodeficiency virus Type 1 (HIV-1) integrase by anti-HIV plant proteins MAP30 and GAP31. Proc Nat'l Acad Sci U.S.A.1995;92:8818-8822.

Li Y-X, Keizer J, Stojilkovic SS, Rinzel J. Calcium excitability of the ER membrane: an explanation for IP$_3$-induced Ca^{2+} oscillations. Am J Physiol, Cell Physiol 1995; 38:C1079-1092.

Li H, Chen HC, Huang FL. Identification of a rapidly dephosphorylating 95 kDa protein as elongation factor 2 during 8-Br-cAMP treatment of N1E115 neuroblastoma cells. Biochem Biophys Res Comm 1995;217:131-137.

Luu NX, Driscoll WJ, Strott CA. Molecular cloning and expression of a guinea pig 3-hydroxysteroid sulfotransferase distinct from chiral-specific 3α-hydroxysteroid sulfotransferase. Biochem Biophys Res Commun 1995;217:1078-1086.

Mahoney CW, Seki K, Huang K-P. Phosphorylation of MARCKS, neuromodulin, and neurogranin by protein kinase C exhibits differential responses to diacylglycerols. Cellular Signalling, 1995;7:679-685.

Mahoney, CW, Pak J, Huang K-P. Nitric oxide modification of neurogranin: identification of the cysteine residues involved in intramolecular disulfide bridge formation using site-directed mutagenesis. J Biol Chem, 1996; in press.

Martìnez A, Miller M-J, Catt KJ, Cuttitta F. Adrenomedullin receptor expression in human lung and in pulmonary tumors. (in press).

Mores N, Krsmanovic LZ, Catt KJ. Activation of LH receptors expressed in GnRH neurons stimulates cyclic AMP production and inhibits pulsatile neuropeptide release. Endocrinology (in press).

O'Malley BW, Strott CA. Steroid hormones: metabolism and mechanism of action. In: Yen SSC, Jaffe RF, Barbieri RL, eds. Reproductive Endocrinology, 4th Ed. WB Saunders (in press).

Seki K, Sheu, F-S, Huang K-P. Binding of myristoylated alanine-rich protein kinase C substrate (MARCKS) to phosphoinositides attenuates the phosphorylation by protein kinase C. Arch Biochem Biophy, 1996;326:193-201.

Sheu F-S, Mahoney CW, Seki, K, Huang, K-P. Nitric oxide modification of rat brain neurogranin affects both the phosphorylation by protein kinase C and the affinity for calmodulin. J Biol Chem, 1996; in press.

Shen L, Huang K-P, Chen H-C, Huang FL. Molecular cloning and characterization of a novel casein kinase II substrate, HASPP28, from rat brain. Arch Biochem Biophy, 1996;327:131-141.

Stojilkovic SS. Calcium signaling in pituitary cells. Trends Endocrinol Metab 1996 (in press).

Stojilkovic SS. Endothelin receptors and gonadal function. Eur J Endocrinol 1996 (in press).

Stojilkovic SS. Measurement and manipulation of intracellular ions. Endocrine News 1996 (in press).

Stojilkovic SS, Catt KJ. Expression and signal transduction pathways of endothelin receptors in neuroendocrine cells. Front Neuroendocrinol 1996; 17:327-369.

Stojilkovic SS, Catt KJ. Novel aspects of GnRH-induced intracellular signaling and secretion in pituitary gonadotrophs. J Neuroendocrinol 1995; 7:739-757.

Stojilkovic SS, Catt KJ. Receptors and secretory actions of endothelins in neuroendocrine cells. Front Endocrinol 1995; 15: 135-144.

Stojilkovic SS, Zheng L, Tomic M, Cesnjaj M, Jobin RM, Catt KJ. GnRH-induced intracellular signaling in pituitary gonadotrophs. The Second International Symposium on Fertility Regulation 1995 (in press).

Strott CA. Steroid sulfotransferases. Endocr Rev (in press).

Tomic M, Jobin RM, Vergara LA, Stojilkovic SS. Expression of purinergic P_2 channels and their role in calcium signaling and hormone release in pituitary gonadotrophs. J Biol Chem 1996 (in press).

Tomic M, Stojilkovic SS. GnRH-induced calcium and current oscillations in gonadotrophs. Trends Endocrinol Metab 1996 (in press).

Tsai-Morris C-H, Buczko E, Geng Y, Gamboa-Pinto A, Dufau, ML. The genomic structure of the corticotropin releasing factor receptor. J Biol Chem 271:14519-14525, 1996.

Scaldaferri L, Arora K, Lee SH, Catt KJ, Moretti C. Expression of PACAP and its type-I receptor isoforms in the rat ovary. Mol Cell Endocrinol 1996;117:227-232, 1996.

Shen L, Huang KP, Chen HC, Huang FL. Molecular cloning and characterization of a novel casein kinase II substrate, HASPP28, from rat brain. Arch Biochem Biophys 1996;327:131-141.

Spergel, DJ, Catt, KJ, Rojas E. Immortalized GnRH neurons express large-conductance calcium-activated potassium channels. Neuroendocrinology 1996;63:101-111.

Stojilkovic SS, Catt KJ. Novel aspects of GnRH-induced intracellular signaling and secretion in pituitary gonadotrophs. J Neuroendocrinol 1995;7:739-757.

Stojilkovic SS, Catt KJ. Receptors and secretory actions of endothelins in neuroendocrine cells. Front Endocrinol 1995;15:135-144.

Stojilkovic SS, Zheng L, Tomic M, Cesnjaj M, Jobin RM, Catt KJ. GnRH-induced intracellular signaling in pituitary gonadotrophs. The Second Internal Symposium on Fertility Regulation 1995 (in press).

Tian Y, Balla T, Bernstein KE, Sandberg K, Baukal A, Catt KJ. Properties of AT_{1a} and AT_{1b} angiotensin receptors expressed in adrenocortical Y-1 cells. Am J Physiol 1996;270:E831-E839.

Venkatachalam KV, Strott CA. Human PAPS synthetase: a bifunctional protein that catalyses formation of the universal sulfonate donor molecule. Gene (in press).

Zhang R., Buczko E, Dufau ML. Cysteine Residues in exons 1 to 6 of the extracellular domain of the luteinizing hormone receptor are required for hormone binding. J Biol Chem 271:5755-5760,?" 1996.

Zhang R, Cai, H, Fatima N, Buczko E, Dufau ML. Functional glycosylation sites of the rat luteinizing hormone receptor required for hormone binding. J Biol Chem 270:21722-21728, 1995.

ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH

Chief

Kevin J. Catt, M.D., Ph.D.

Senior Investigators

Maria L. Dufau, M.D., Ph.D
Kuo-Ping Huang, Ph.D.
Charles A. Strott, M.D.

Tenure Track

Stanko Stojilkovic, Ph.D.

Staff Scientists

Freesia Huang, Ph.D.
Young Ja Lee, Ph.D.
Chon-Hwa Tsai-Morris, Ph.D.

Facility Manager

Hao-Chia Chen, Ph.D.

Senior Fellows

Krishan Arora, Ph.D.
Tamas Balla, M.D., Ph.D.
Azra Khanum, Ph.D.
Lazar Krsmanovic, Ph.D.

Post-Doctoral Fellows

Harukuni Akita, M.D., Ph.D.
Gabrielle Bonnomme, Student
Zhengyi Cheng, M.D., Ph.D.
Gregory Downing, M.D., Ph.D.
Yi Geng, Ph.D.
Xioabin Guan, M.D., Ph.D.
Zhang-Zhi Hu, Ph.D.
Wen-Ling, Hsieh, Ph.D.
Hong Ji, M.D.
Richard Jobin, Ph.D.
Li Junfa, M.D.
Taka-aki Kohlmizu, M.D., Ph.D
Agnieszka Lachowicz, M.D., Ph.D
Yashushi Miyagawa, M.D.
Nadia Mores, M.D., Ph.D.
Hiroshi Murakami, M.D.
Jhang Ho Pak, Ph.D.
Kang Rao, M.D.
Sai Xiao, M.D., Ph.D.
Li Shen, Ph.D.
Shingo Takada, M.D., Ph.D.
Pei-Zhong Tang, M.D.
Ying Tian, M.D.
Melanija Tomic, Ph.D.
Tayuka Tomizuka, M.D., Ph.D.
Misook Uh, Ph.D.
Kallidaikurichi Venkatachalam, Ph.D.
Jian Wang, M.D., Ph.D.
Wang Ziaoming, M.D.
Ran Zhang, Ph.D.
Meng Zhang, M.D.
Li-Xin Zheng, M.D.
Li Zhuang, M.D.

Pre-Doctoral Fellows

Michael Lee
Nga Luu
Xueo Zhao

Guest Researchers

Hitoshi Chiba, M.D.
Jagadeesh Gowraganahalli, Ph.D.
Hiromi Miyagawa, M.D.
Suguru Saito, M.D.
Philippe Selvais, M.D.
Roger Smith, Ph.D.
Annamaria Zolyomi, M.D.
Ann Chen Katzur, Student

Senior Technical Specialists

Albert Baukal
John Morrell

Project Numbers - Annual Report 1996

ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH (ERRB)

ZO1 HD 00035-24	The Structure and Function of Peptides and Proteins Hao-Chia Chen, Ph.D.
ZO1 HD 00150-21	Characterization of Gonadal Receptors and Mechanisms of Action of Peptide Hormones in Steroidogenic Cells Maria L. Dufau, M.D., Ph.D.
ZO1 HD 00184-18	Regulation of Pituitary Hormone Secretion Kevin J. Catt, M.D., Ph.D.
ZO1 HD 00187-17	Hormonal Regulation of Cellular Metabolism Kuo-Ping Huang, Ph.D.
ZO1 HD 00193-11	Angiotensin II Receptors and Activation Mechanisms Kevin J. Catt, M.D., Ph.D.
ZO1 HD 00194-08	Steroid Biosynthesis and Metabolism in the Mammalian Adrenal Cortex Charles A. Strott, M.D.
Z01 HD 00195-03	Intracellular Signalling in Endocrine Cells Stanko Stojilkovic, Ph.D.

ZO1 HD 00196-301

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD00035-24

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Structure and Function of Peptides and Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H.C. Chen	Head, UMSPC	ERRB, NICHD
Others:	K.J. Catt	Chief	ERRB, NICHD
	J.L. Morell	Research Chemist	ERRB, NICHD
	M. Zhang	Visiting Fellow	ERRB, NICHD
	S. Saito	Guest Researcher	ERRB, NICHD

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Unit of Molecular Structure and Protein Chemistry

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project focuses on the expression, immunological properties, and structure-function relationships of peptide hormone receptors. (1) We expressed the full-length and 1-240 residue segment of the rat angiotensin II (Ang II) type 1b receptor (AT1b) in baculovirus/Sf9 insect cell systems as non-fused protein and glutathione-S-transferase fusion proteins. Western blot analysis using a AT1 specific antiserum raised against the C-terminal segment (LE92, residues 268-369) of the AT1b receptor revealed bands ranging from 66-210 kDa. The recombinant proteins were found mostly in the inclusion bodies and none was detected in the soluble fraction or medum, even when a conventional secretory signal sequence was included in the vector. Ang II binding activity was detectable but was lower than that of partially purified bovine adrenal membranes (BAM). (2) The human AT_1 receptor was expressed using full length cDNA from a plasmid vector containing a 6XHis tag, and an baculovirus vector/cabbage looper cell system. Ang II binding analysis of the optimally infected cells showed 90 times more binding sites than those found in the BAM on a protein weight basis. However, the majority of the receptor protein was found in the inclusion bodies, with bands in the 66-210 kDa range as revealed by Western blot analysis using anti-LE92 serum. It is probable that the receptors expressed on the plasma membrane are those that display hormone-binding activity. Purification of the receptor protein by Ni-affinity chromatography is in progress. (3) In addition to seven chemically synthesized segments of the GnRH receptor, three long segments (residues 19-77, 138-194, and 265-359) each fused with maltose-binding protei were expressed in *E. coli*. Milligram quantities of the fusion proteins were obtained after maltose-affinity chromatography, and were used to generate antibodies in rabbits. After Factor Xa cleavage, the fragments yielded N-terminal amino acid sequences consistent with those of the respective receptor fragments.

Project Description

Objectives:

This project is concerned with the analysis, synthesis, and structure-function relationships of biologically active peptides and proteins with particular emphasis on the study of structural and physico-chemical characteristics in order to elucidate structural requirements for various biological activities. Our current primary research objectives are placed on the structure and function of angiotensin II and gonadotropin releasing hormone receptors which include:
(1) chemical synthesis of peptide fragments derived from protein sequences of the two receptors,
(2) high yield expression of full length and partial sequences of the receptor molecules,
(3) generation of specific antibodies against the two receptors,
(4) immunological characterization and localization of the receptors in tissues and cultured cells,
(5) biochemical and physico-chemical characterization of receptor proteins.

We are also undertaking long term collaborative projects involving the identification and structural determination of novel protein kinase C substrates in the brain, and the identification of hormone and neuropeptide processing enzymes and determination of their specificities of cleavage.

Methods Employed:

A wide variety of advanced techniques for peptide and protein chemistry were employed. These include amino acid microanalysis, protein sequencing, peptide synthesis, electrophoresis, spectropolarimetry, and conventional and high performance liquid chromatography. Special methods using novel protecting groups to meet the requirement of highly selective removal in peptide synthesis and specific chemical techniques for the modification and cleavage of amino acids in a peptide chain were designed and employed. PCR, Western blot analysis, immunocytochemical analysis, *in situ* hybridization, ribonuclease protection assay and photoaffinity labeling of receptors are the major techniques employed in molecular biological investigations.

Major Findings:

Protein Expression of Rat Angiotensin II Receptor Type 1b. Our initial attempts to obtain high-yield full length AT_{1b} receptor and its segments by two bacterial systems using PinPoint and pMAL vectors resulted in a limited success allowing only the expression of the carboxyl-terminal segment LE92 (residues 268-359). We therefore initiated the expression of the full-length receptor and its 1-240 segment in an attempt to use the recombinant proteins as antigens to generate sufficiently high avidity antibodies for immunological characterization of the receptor protein in tissues. Also, to use the receptor molecule to conduct biochemical and structural characterization, and in particular to investigate post-translational modifications. Genes coding for the full length AT_{1b} (1.08 kb) and for amino acid residues 1-240 (0.72 kb) were cloned separately into selected baculovirus transfer vectors from PharMingen. A vector containing 1.3 kb of AT_{1b} (designated as DraIII-pcDNAI) that was previously constructed in the Branch was used for fragmentation and amplification to obtain 1.08 kb and 0.72 kb gene sequences. In order to increase the level of expression, toth the 1.08 kb gene and DraIII (1.3 kb) gene were cloned into pVL1393 plasmid (PharMingen), resulting in the expression of a non-fused protein. In addition, the 1.08 gene was cloned into two plasmids, namely pAcG2T and pAcSecG2T also from PharMingen. These two systems allowed expression of a GST fusion protein. The difference between two GST systems is that pAcSecG2T has an in-frame gp67 signal sequence preceding the GST gene to allow secretion of the fusion protein into the supernatant. The signal sequence will be cleaved off during secretion. Similarly, the 0.72 sequence was also cloned into the two transfer vectors using the same strategies as for the full-length construct.

Each of the seven constructs was co-transfected with linearized baculovirus DNA (Baculogold^{TM}) in to *Spodoptera frugiperda* Sf9 cells by calcium phosphate co-precipitation. The virus amplification was continued for five days before collecting the supernatant which contains the recombinant baculovirus. Sf9 cells (2 x 10^7 per 15 cm plate) infected with high titer recombinant baculoviruses were cultivated for 3 days. The lysates from transfected cells with four full-length AT_{1b} genes showed dense multiple bands corresponding to 66 kDa to 210 kDa by Western blot analysis using the antiserum against LE92. In a parallel analysis, the same amount of bovine adrenal membrane yielded only a faint band at 66 kDa. Similar results were obtained from lysates of 1-240 transfected cells using an antiserum against the N-terminal peptide (residue 1-26). The antiserum against LE92 was completely inert. However, these same four transfected cells containing full-length cDNA displayed Ang II binding activity lower than that of the adrenal membrane. Purification of these GST fusion proteins by glutathione affinity chromatography had only limited success. The high contents of AT_{1b} protein in the cells but poor Ang II binding activity indicate that the majority of the expressed protein is in the inclusion bodies. Thus, it became apparent that a denaturant such as urea or guanidine hydrochloride would have to be employed in order to extract the protein and to refold it in an attempt to generate an active form. However, such conditions make it impossible to take advantage of GST in establishing an effective affinity chromatographic system. We therefore changed our strategy from using GST to "Histag" as described in the following section.

High-Yield Expression of Human Angiotensin Receptor Type 1. Due to the difficulty described in the preceding section with regard to purification of the receptor protein granulated as the inclusion bodies, and with the advent of innovations in baculovirus protein expression systems, including recombinant viral plasmid (pFastBac 1; Life Technologies), host insect cells (cabbage looper *Trichoplusia ni*, also known as High Five^{TM} from Invitrogen), we have carried out the expression of the human receptor with an improved experimental design. Studies of the human receptor has an advantage of future clinical application in addition to gaining basic knowledge in receptor biology. Unlike rodent species, only one Type 1 receptor (AT_1) has been found in the human. A gene encoding human AT_1 in the form of hATR1-PcDNA I obtained from SmithKline Beecham, Inc. was used to construct a recombinant donor plasmid in pFastBacHTb plasmid from Life Technology. The AT_1 sequence is preceded with six consecutive histidines (6XHis), a spacer and an rTEV protease cleavage site. The recombinant bacmid DNA generated from *E. coli* (*Lac7*$^-$) was transfected into High Five^{TM} (Invitrogen) insect cells. This cell line has been favored because the cells are larger and have efficient post-translational modification machinery. Also, the cells can be adequately grown in serum-free media. Hormone binding analysis of the optimally infected cells using the [Sar^1,Ile^8]Ang II antagonist detected 90 times more binding sites than those found in the purified bovine plasma membrane, on a protein weight basis. This level of expression is a significant improvement over all known natural sources. However, the majority of the receptor protein was found in the inclusion bodies, as revealed by Western blot analysis using antiserum against the LE92 fragment of the rat AT_{1b} receptor. Moreover, multiple bands ranging from 66-210 kDa were also found. These results indicate that the receptors are distributed mostly inside the inclusion bodies and that those on the plasma membrane display hormone binding activity. Since the expressed protein carries 6XHis, it was solubilized in the PBS buffer containing 6 M guanidine chloride. Purification of the protein by Ni-affinity chromatography is now in progress, large scale production has been initiated.

Chemical Synthesis and High-Yield Expression of GnRH Receptor Segments. Based on the cDNA of murine gonadotropin-releasing hormone (GnRH) receptor deduced amino acid sequence, seven segments from different domains in the receptor protein sequence were selected and synthesized by the solid phase method. They were designated as GnRH1/12 (Res. 316-327, in putative seventh transmembrane domain and C-terminal), GnRH2/19 (Res. 248-266, in third cytoplasmic domain) GnRH3/22 (Res. 14-35, first extracellular domain), GnRH4/42 (Res. 229-270, all third cytoplasmic domain), GnRH5/20 (Res. 178-197 in third extracellular domain), GnRH6/16 (Res. 291-306, fourth extracellular domain) and GnRH7/30, Res. 105-121 & 186-198, the second and third extracellular domains cross-linked between two inner Cys residues). Two more peptides from the cytoplasmic domain were also synthesized, and were designated as GnRH8/25 (Res. 58-82) and GnRH9/25 (Res. 134-158), respectively. The peptides were conjugated to thyroglobulin through Sulfo-SMCC, a heterobifunctional linker, via added cysteine on the N-terminus of the peptide. Rabbits immunized with these conjugates produced appreciable antibody titers. However, the antibodies are of low avidity and not sufficiently specific for the immunological characterization of the receptor protein. In order to generate antibodies of improved avidity, a rational approach by increasing the size of antigen is considered. We therefore undertook an approach to express the protein in segments consisting of extracellular, transmembrane, and cytoplasmic domains. We have obtained mg quantity of three maltose-binding protein (MBP) fusion proteins containing the segments corresponding to amino acid residues 19-77 (designated G19), 138-194 (G138) and 292-327 (G292) 265-359 of the receptor. These have been expressed in *E. coli* strain TB1 transfected with a plasmid with the corresponding gene inserted downstream from the *mal*E gene in the pMAL-2c (Biolabs) vector, which also contains the sequence coding for the recognition site of the specific protease Factor Xa located at the 5' insertion site. The *mal*E gene encodes maltose-binding protein, resulting in the expression of an MBP fusion protein that allows one-step purification through maltose-affinity chromatography. Upon purification of fusion proteins from the cell lysate by maltose-affinity chromatography and cleavage of the fusion proteins with Factor Xa, fragments of the projected sizes as revealed on SDS-PAGE were obtained. An optimal cleavage condition (Enzyme:protein =1:20 by wight, 4°C, 48 hrs.) was established that allows more than 75% efficiency of cleavage without detecting other internal cleavage by N-terminal sequencing analysis. Furthermore, the fragments yielded an N-terminal amino acid sequences consistent with the sequences of respective fragments. For the generation of antibodies, the MBP fusion proteins have been used to immunize rabbits.

Significance to Biomedical Research and the Program of the Institute:

We have obtained a new antiserum generated against a 92 residue C-terminal segment of angiotensin (Ang II) receptor type 1b that recognizes by the Western blot analysis a 66-67 kDa protein in bovine adrenal plasma membrane and cells, rat tissue homogenates of adrenal, liver, brain and uterus but not heart or spleen. This finding is consistent with the data from Ang II binding experiments. The photoaffinity labeling of bovine adrenal plasma membrane and rat tissue homogenates with ^{125}I-[Sar^1,(4-N$_3$)Phe^8]Ang II and immunoprecipitation with this antiserum revealed also a 66-67 kDa species. The new antiserum raised agaonst LE92/MPB recognizes the AT1 receptor with improved specificity, and will be useful for immunocytochemical study of receptor localization and for Western blot analysis of the expression and biochemical characterization of the receptor. Its ability to inhibit Ang II binding to the receptor, and subsequent biochemical responses, will also be evaluated.

In the same line, three fragments from murine GnRH receptors have been over-expressed. Antisera against these domains will be essential for immunocytochemical study of the receptor localization in tissues, and for Western blot analysis for the expression and biochemical characterization of the GnRH receptor. GnRH plays a pivotal role in LH and FSH secretion of the pituitary gland. Determination of the structure of the GnRH receptor may lead to an understanding of the molecular mechanism of receptor activation and regulation. Although the amino acid sequence deduced from GnRH cDNA suggests seven putative transmembrane domains characteristic of G-protein-coupled receptors, it allows the assignment of a very short intracellular C-terminal segment lacking a typically polar sequence.

In addition to the expression of the receptor fragments, we have achieved a certain degree of success in the high level expression of whole rat AT_{1b} and human AT_1 angiotensin II receptors in baculovirus systems. Although some difficulty will be encountered in order to solubilize, purify, and reconstitute the protein into an active form, the incorporation of a 6XHis residues into the receptor sequence provides a great advantage for the selection of conditions for solubilization and purification. As soon as mg quantity of the receptor protein can be obtained, regardless of its level of hormone-binding activity, it will be useful to generate both polyclonal and monoclonal antibodies for the purpose of immunological and biochemical characterization of the receptor.

Proposed Course:

We will continue our efforts on the purification and characterization of antibodies specific to either Ang II or GnRH receptors, and studies on the tissue distribution, expression, glycosylation, and signal transduction of these two receptors. Tissue distribution and expression of these two receptors will be studied in fetal, infant, and adult rats under the influence of various physiological and pathophysiological conditions. This area of research will be vigorously pursued in light of the recent availability of confocal electron microscopy in the Branch. High yield expression of both human AT_1 and mouse GnRH receptors and their fragments will continue with particular emphasis on the His-tagged GnRH receptor expression using the baculovirus system used for human AT_1. Purification of receptor proteins using ligand-affinity and immunoaffinity chromatography will also be pursued.

In addition to immunological characterization of these two receptors in tissues, physico-chemical, functional, and immunological characterization of recombinant receptors will be studied. These include:

(1) Ligand-binding activities and molecular weight determination.
(2) Post-translational modifications of the receptor: glycosylation and phosphorylation.
(3) Characterization of solubilized receptor: ligand-binding activities and molecular weight.
(4) Purification of solubilized receptor.
(5) Identification of glycosylation, phosphorylation, and hormone binding sites.
(6) Determination of disulfide linkages.

Publications:

Lee-Huang S, Huang PL, Huang PL, Bourinbaiar AS, Chen HC, Kung SF. Inhibition of human immunodeficiency virus Type 1 (HIV-1) integrase by anti-HIV plant proteins MAP30 and GAP31. Proc Nat'l Acad Sci U.S.A.1995;92:8818-8822.

Ehrlich A, Barnett VA, Chen HC, Schoenberg M. The site and stoichiometry of the N-phenylmaleimide reaction with myosin when weakly-binding crossbridges are formed in skinned rabbit psoas fibers. Biochim Biophys Acta 1995; 1232:13-20.

Li H, Chen HC, Huang FL. Identification of a rapidly dephosphorylating 95 kDa protein as elongation factor 2 during 8-Br-cAMP treatment of N1E115 neuroblastoma cells. Biochem Biophys Res Comm 1995;217:131-137.

Shen L, Huang KP, Chen HC, Huang FL. Molecular cloning and characterization of a novel casein kinase II substrate, HASPP28, from rat brain. Arch Biochem Biophys 1996;327:131-141.

Cawley NX, Chen, HC, Beinfeld MC, Loh YP. Specificity and kinetics studies on the cleavage of various prohormone mono- and paired basic residue sites by yeast aspartic proteases 3. J Biol Chem 1996;271:4168-4176.

Johnson BE, Damadaran A, Rushin J, Gross A, Le PT, Chen HC, Harris RB. Ectopic production and processing of atrial natriuretic peptide in a small cell lung cancer cell line and tumor from a patient with hyponatremia. Cancer (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00150-21-ERRB

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of Gonadal Receptors and Mechanisms of Action of Peptide Hormones in Steroidogenic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Maria L. Dufau	Chief, SME	ERRB, NICHD
Others:	C.-H. Tsai-Morris	Chemist GS-13	ERRB, NICHD
	Azra Khanum	Visiting Scientist	ERRB, NICHD
	Z.Z. Hu	IRTA Fellow	ERRB, NICHD
	Y. Geng	IRTA Fellow	ERRB, NICHD
	L. Zhuang	NRSA Fellow	ERRB, NICHD
	Y. Miyagawa	Visiting Fellow	ERRB, NICHD
	P-Z. Tang	Visiting Fellow	ERRB, NICHD
	H. Murakami	Visiting Fellow	ERRB, NICHD

COOPERATING UNITS *(if any)*

Contract for preparation of gonadal cells and cell fractions HD-6-2904

LAB/BRANCH
Endocrinology and Reproduction Research Branch

SECTION
Section on Molecular Endocrinology

INSTITUTE AND LOCATION

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1) The luteinizing hormone receptor is a seven transmembrane G protein-coupled receptor with a high-affinity hormone binding site located on its N-terminal extracellular domain. Mutational studies have identified two specific classes of extracellular cysteine residues that are required for surface expression or hormone binding. All four non-conserved cysteines from exon 1 are essential for binding activity and the conserved cysteines in exon 5 and 6 (109,134) are important for hormone binding. Disulfide bonding among cysteines in exon 1, and between those of exon 5 and 6, were indicated by these studies. All but two of the eight remaining cysteines, including those in loops 1 and 2 (exons 7-11) produced defects in receptor translocation to the cell surface but not in hormone binding. Thus, the requirement of cysteine in exon 1 for hormone binding activity is not related to processing of the nascent receptor. The exon 1 cysteines, in conjuction with the N-glucosamine residue of the glycosyl chains at Asn152 and Asn173, are required for refolding of the hormone binding domain of the denatured/reduced mature receptor to its active configuration. The sugar residues are required for stabilization/acquisition of the active conformation of the mature receptor and of the nascent receptor. 2) Isolation and structural characterization of the corticotropin-releasing hormone gene included the complete mapping of its intron/exon organization and the basis of the pituitary splice variants, and the isolation of novel spliced variants of the Leydig cell CRF receptor. These studies suggest that the differences in signal transduction between pituitary and Leydig cells are not attributable to the expression of different receptor isoforms. They also provide evidence for an evolutionary link between the intronless transmembrane/cytoplasmic module of the glycoprotein receptors and the intron-containing module of the CRF receptor. 3) Unravelling the complex structure of the prolactin receptor gene has identified promoter regions and regulatory elements involved in transcriptional activation of the tissue-specific promoters (PI-PIII). The promoter utilized in the mammary gland has been identified. 4) CYP17, the key enzyme of the androgen pathway, was found to have species and tissue-specific differences in its regulatory sequences. Such differences in the amino acid domains required for hydroxylase activity within and between species (human/bovine vs. rat) are consistent with their differential regulation of lyase activity in steroidogenic cells.

Project Description:

Objectives:

A. Gonadal receptors: structure, regulation, and function.

(1) Investigation of physical, molecular and functional characteristics of gonadotropin and prolactin receptors, including gene structure, expression, and translation; (2) Overexpression of LH and FSH receptors, and development of scale-up procedures for purification of receptors for structural analysis; (3) Analysis of the relationship between receptor sites and adenylate cyclase enzyme complex; and (4) Investigation of membrane protein phosphorylations as intermediate steps in hormone action.

B. The androgen pathway: hormonal regulation of testicular function.

(1) To define the mechanisms of action of trophic hormones upon steroidogenesis and in the broadest sense the modulatory action of peptide hormones and growth factors upon development and functions of the Leydig cell. (2) To study cell-to-cell communication within the interstitium and the functional interactions of the Leydig cell with the tubule compartment and *vice versa.*

Methods Employed:

A. Gonadal receptors: structure, function, and regulation.

Assay of particulate and soluble receptors by ligand-binding and centrifugation, millipore filtration and polyethylene glycol precipitation; adenylate cyclase assay; affinity chromatography; SDS gel electrophoresis. Electroblotting; double dimension gel electrophoresis. Phosphorylation of membrane proteins and purified receptors. Cholera toxin and pertussis toxin-catalyzed ADP ribosylation. Immunization of mice and rabbits with purified receptors; fusion, screening, production of monoclonal and polyclonal antibodies to receptors; microsequence analysis of lactogen and LH receptor N-terminal regions and protein fragments. Peptide synthesis, DNA synthesis, construction of DNA probes, screening genomic libraries, cloning procedures, DNA sequencing. Subcloning of receptor cDNA, wild type receptor fragments and mutated forms in expression vectors. Expression of receptors in COS cells, Y_1 cells, and CHO and HeLa cells. Nb_2 lymphoma cell culture, bioassay of lactogenic hormones. Overexpression of receptors in insect SF9 and high-five cells. Site-directed mutagenesis of cDNA and genomic fragments by RCPCR. Subcloning of 5' and 3' flanking regions of the LH and prolactin receptor genes in CAT vector and Luciferase vectors. CAT and Luciferase assays, gel shift analyses and foot printing. Isolation of specific transacting factors; nuclear transcription assays; Preparation of 5' flanking and promoter and gene fragmensts wild type and mutants of the LHR and PRLR/luciferase incorporated into the genome of a replication defective human adenovirus vector by homologous recombination for infections of primary Leydig cells, luteal and folicular cells at different stages of development.

B. The androgen pathway: hormonal regulation of testicular function.

Preparation of Leydig cells by collagenase digestion. Purification of Leydig cells by centrifugal elutriation and metrizamide gradients. Electron microscopy, light microscopy and autoradiography. Characterization of LH, GnRH, CRF, angiotensin II, and serotonin receptors by binding analysis in cell membrane and intact Leydig cells. Primary culture of Leydig cells and testis explants. Development of antibodies to CYP17 in rabbits. Steroid analysis of incubation media by specific radioimmunoassays and of peptides and cyclic nucleotides. Measurements of CYP17 steroidogenic enzyme activity (17α-hydroxylase/17-20 desmolase activity) in transfected cells with wild type and mutant cDNA by conversion of steroid precursors of $\Delta 4$ and $\Delta 5$ pathways. Measurement of CYP17 concentrations in Leydig cell microsomal fractions by specific radioimmunoassay. Assay of 17β-hydroxysteroid dehydrogenase activity in rat Leydig cells. Preparation of mRNA from adult and fetal Leydig cells. Transcriptional assays, Northern blot analysis, dot and slot blots, hybridization of blots with 17α-hydroxylase/17 desmolase cDNA probes. Hybridization histochemistry of fetal cell cultures. Screening of rat testis and ovarian libraries and genomic library, DNA sequencing. Transfection of mammalian cells in continuous culture using DOTAP or lipofectamine. Preparation of rat CYP17 wild type promoter and mutants/luciferase fusion genes incorporated into the genome of a replication defective human adenovirus vector by homologous recombination for infections of primary Leydig cell (expressing) and adrenal cell cultures (non-expressing). DNA sequencing. Site-directed mutagenesis using polymerase chain reaction. 17β-hydroxysteroid dehydrogenase (HSD) activity in intact Leydig cells. Cloning of 17β-HSD from Leydig cell expression libraries, and with probes from the HSD type 3 human cDNA sequence.

Major Findings:

A. Gonadal receptors: structure, regulation, and function.

Studies of Cysteines residues from the extracellular domain and extracellular loops of the LH receptor and their function in hormone binding or surface expression.

The luteinizing hormone receptor (LHR) is a seven-transmembrane G-protein coupled receptor with a high affinity hormone binding site in the N-terminal extracellular domain. This hormone binding domain is composed of two Cys-rich regions in exons 1 and 9, generally conserved in the glycoprotein hormone receptor family and bordering a leucine-rich domain (exons 2-8). A third Cys-rich region (Cys-314, -321, and -331) is located in the region of the extracellular domain, adjacent to the transmembrane module (exon 10) and well outside the binding domain. This region is also conserved in the FSHR and TSHR. Only Cys-109, Cys-134 (exons 5 and 6) and Cys-282 are not conserved in the FSHR and TSHR. In extracellular loops 1 and 2 (EL1, EL2) two Cys are present in LHR which are conserved in most of GPCRs and are of structural importance in the adrenergic/rhodopsin receptor family.

Relevance of Cys residues to receptor binding

To study the relevance of cysteines and disulfide bonding to LHR hormone binding, all extracellular cysteines including those in EL1 and EL2 loops of the holoreceptor (Form A), and the corresponding cysteines of the LHR splice variant form B (that includes exons 1-10 containing extracellular domain and a unique 22 amino acid tail with an additional Cys) were replaced by serine residues. The mutant receptors were expressed in COS1 cells or overexpressed in insect cells. The Ser for Cys substitution prevents formation of a putative disulfide bond without significantly affecting the charge characteristics of the protein. The additional Cys 305 in form B cancels potential unpaired cysteines and serves to reduce intermolecular aggregation that could result from reactivity of an unpaired cysteine. Our results demonstrate that only the four non-conserved Cys from Exon 1 (Cys 8,12,14 and 22) are essential since their individual replacement abolished hormone binding activity. In addition, the two unique Cys at 109 and 134 are of importance since their mutation caused 75% decreases in both cell surface and total cellular solubilized LHR hormone binding. The latter provides evidence for generalized decreases in binding rather than in an individual compartment. The four non-conserved Cys in exon 1 are each essential for hormone binding activity and no other LHR Cys can replace their function in the mutant LHRs. In studies reported by others, mutation of the analogous Cys in exon 1 of the TSH receptor did not affect TSH binding, suggesting that these have a unique function in LH/hCG binding. Since putative disulfide bonds between Cys in exon 1 and Cys 109 or Cys 134 should yield equivalent reductions in activity upon mutation of each half cystine (i.e., no hormone binding), which was not the case in our study, a putative disulfide bond is suggested between Cys-109 and Cys-134 in the hormone binding domain. Ser-134 LHR exhibited a marked reduction in maximal hormonal activation of cAMP in comparison with the wild-type LHR that was consistent with the 75% decrease in binding activity. Similarly, substitution of Cys-109 reduced maximal hormonal stimulation. These single substitution experiments demonstrate that specific cysteines in exons 1-6, perhaps through disulfide interaction, are essential/important for the active receptor configuration.

Importance of Cys residues in surface expression

In this study, we identified another class of cysteines (including extracellular Cys 257, 258, 321, and 331, and the EL1 417 and EL2 492) that are not involved in hormone binding but are essential for surface expression of the LHR. Specifically, these mutations resulted in impaired (Cys 282) or total loss of surface expression (all others) in COS-1 cells transfected with the holoreceptor. However, the binding activity of these mutants was recovered in detergent-solubilized extracts. Other Cys residues, including Cys-282 (exon 10) and Cys-314 (exon 11), were not essential for hormone binding or plasma membrane insertion. Also, the substitution of the function of contiguous Cys 257 and 258 residues in exon 9 was ruled out by double mutation of this residues. In addition, radioimmunoassay studies indicate that while substitution of many of the LHR cysteines resulted in retention of the mutant proteins in the nonsurface membrane fraction, no changes in steady-state concentrations of the receptor were observed. The presence of the correct disulfide configuration necessary to initiate signal transfer may be a prerequisite for membrane insertion, but acquisition of the high-affinity binding conformation is not.

The relevance of disulfide bonds to hormone binding

The importance of disulfide bonds in hormone binding activity was investigated in ligand blots using the form B spliced variant overexpressed in insect cells and the affinity-purified Form A holoreceptor isolated from ovarian membranes. Reduction and boiling of the 38 kDa form B receptor prior to electrophoretic separation and transfer to nitrocellulose did not reduce the ability of the electroblotted receptor to bind labeled hCG. These findings demonstrate that the initial oxidation state does not affect the ability of the Form B LHR to renature to the active binding conformation during transfer to nitrocellulose membranes. They also indicate that renaturation of the isolated form B binding domain involves the reformation of exon 1 disulfide bonds, since the primary impact of the conservative substitution substitution of Ser for Cys is in the loss of the ability to form disulfide bonds.

In contrast, oxidation and denaturation of the full length (form A) receptor severely impaired its ability to bind hormone after electrophoresis and ligand blotting. Thus, the presence of the transmembrane/cytoplasmic domains presumably has a negative effect on renaturation to the active binding conformation of the reduced LHR on nitrocellulose. In the form A holoreceptor, reactive cysteines from transmembrane or cytoplasmic domain, exposed only under reducing conditions, may interfere with these putative disulfide interactions in the extracellular hormone binding domain and impair ligand binding.

Our previous studies using enzymatic digestion showed that folding of the nascent Form B LHR receptor that lacks the transmembrane cytoplasmic domain to an active configuration is dependent on the presence of N-linked carbohydrates at Asn173,152, specifically the N-acetylgalactosamine of the chain attached to asparagine. In more recent studies we have established that the LHR form B receptor is only capable of spontaneous renaturation during ligand blotting, following SDS denaturation and boiling in the presence or absence of reductant, if the receptor bears the proximal N-acetylglucosamine on Asn173 and Asn152 and the four cysteines in exon 1.

Structural characterization of the rat corticotropin releasing factor receptor gene - isolation of CRF receptor isoforms.

The previous identification of several variants of the CRF receptors that carry insertions and deletions of both the extracellular and seven-transmembrane module suggests the presence of multiple introns in the CRF receptor gene, and the expression of splice variants that potentially may differ in ligand specificity and modes of signal transduction. These findings by others were relevant to our observation that the CRF receptor which we identified in the rat Leydig cell, unlike the pituitary receptor, did not stimulate cAMP production and did not appear to be coupled to G_s. Activation of this receptor through a non-cAMP mediated mechanism inhibits the stimulatory actions of LH at the level of adenylate cyclase. This unconventional coupling of the CRFR suggested a tissue-specific post-translational modification of the CRFR, different G proteins or inhibitors, or the presence of a splice variant of the CRF receptor in the Leydig cell. The structural organization of the CRF receptor gene was determined for subsequent elucidation of isoforms in order to clarify the nature of the Leydig cell receptor.

Isolation of the CRF receptor gene

The rat CRF receptor gene was isolated from a rat liver lambda dash genomic library using rat pituitary CRFR cDNA (form A-pituitary full-length form). The CRFR gene contains 13 exons and 12 introns, and the positions of the exon/intron junctions are similar to those of other Class II G protein-coupled receptor genes including the parathyroid hormone and glucagon receptors. Exons 1-4 correspond to the extracellular domain and exons 5-12 the transmembrane and exon 13 cytoplasmic module. The TATA-less CRFR promoter resides within 593 base pairs of the initiation codon and the major transcriptional start site at nucleotide -238, approximately 20 bp downstream of an AP2 site and 60 bp of an SP-1 consensus element. The addition of the domain between -593 and -651 increased promoter activity in two cell types, indicating that an enhancer or a second promoter is located between -593 and -651. Intron junctions were identified in the extracellular, transmembrane, and cytoplasmic domains of the CRFR, giving the potential for differential signal transduction by splice variants.

CRFR receptor variants - CRFR cDNA isolated from the rat Leydig cell

The reported CRF receptor cDNA variants were found to correspond to the deletion of complete exons. Exon 3 of the extracellular domain is deleted from a human variant 3. This deletion is in frame and does not result in a premature termination codon. A second human variant, the CRF Type 2 receptor, contains an 87-nucleotide insertion after exon 5 of the rat CRF receptor gene. This insertion was not contained within the rat intron between exon 5 and 6, indicating that this specific human variant is not found in the rat. The Leydig cell CRFR mRNAs were expressed at very low levels in comparison with the pituitary, and the predominant form of the Leydig cell was identified as the rat pituitary CRF receptor sequence form A. Two splice variants (B and C) were also recovered from rat Leydig cell mRNA but at very low expression levels. The first variant form B recovered by PCR contains a deletion of exon 3 that was previously observed in the human 3 receptor isoform. We have not yet identified the positions of the initiation and termination codons in CRFR form B (Leydig cell), but our studies indicate that the signal sequence of this clone (exon 1) differs from that of the Form A CRFR. A second rat Leydig cell variant, CRFR form C cDNA, was amplified with a 5' primer begining in exon 3 at position 215 and continues to exon 13 with deletions of exons 7, 11, and 12. Exons 1 or 2 were not amplified with form A primers, indicating a novel amino terminal sequence. The PCR product begins with amino acid 73 in exon 3. Deletion of exon 7 results in a frameshift with premature termination after a unique 39 amino acid tail that retains two hydrophobic transmembrane domains. It remains to be proven whether this form of CRFR is responsible for the unique properties of the Leydig cell CRFR. Because the pituitary form is the more abundant among the low-expressed forms of the CRFR in the Leydig cell, it is conceivable that the lack of coupling to G_s is related to receptor density. However, it is also possible that our initial exploration has not detected the relevant form involved in CRF-induced coupling that leads to inhibition of gonadotropin action in Leydig cells.

Similarity of the CRF receptor gene to the glucagon receptor family, and evolutionary considerations

The CRF receptor gene exhibits substantial similarity to the glucagon/PTH receptor gene family, and is characterized by the presence of introns within its transmembrane/cytoplasmic module, highly conserved cysteines in its extracellular domain, and a highly conserved extracellular loop. The CRF receptor exon/intron junctions are aligned with those of the PTH and glucagon receptors after exons 3,5,7,8,9,10, and 12. Exons 11 and 12 are divided in CRFR and PTHR whereas this domain in the glucagon receptor is a single exon. All members of this receptor family stimulate cAMP generation and phosphoinositide hydrolysis, consistent with the similarity of their TM domains and intracellular loops.

The presence of an alternative acceptor splice site at position 1895 within the intronless LHR exon 11 module to produce a splice variant, initially identified in the pig as Form D, has provided insights into the origin of the intron-containing CRFR TM/C module, because it is positioned at the same location as intron 12 of the CRFR, immediately following TMVII. The alternative splice sites in the LHR coding region substitute for the 3' of the intron, serve as a cleavage site and possibly as a site of intron insertion in evolution. The 3' element of the CRFR exon 11 conforms to the LHR TTXCAG 3'intron J splice element and the alternative splice sites that produce LHR forms B and C. This position is not an intronic site in the glucagon receptor but is in the PTHR and CRFR. Thus, the glucagon receptor may be an intermediate form between the LHR and CRFR. CRF exons 1,4,5,10,11 and 12 terminate in the 3' intron acceptor consensus element AG, indicating that these positions may served as alternate acceptor or intron insertion points. Thus, from these studies it is proposed an evolutionary mechanism of CRF Class 2 intron insertion in evolution derived from the intronless exon 11.

Multiple and tissue-specific control of the prolactin receptor gene in the rat gonadas and liver.

We have recently succeeded in cloning the rat prolactin receptor gene, and have identified tissue-specific promoters that control the expression of the receptor in specific target tissues including the liver and gonads and mammary gland. The expression of the rat prolactin receptor gene is controlled by at least three promoters, PI, PII, and PIII, which precede non-coding exons $E1_1$, $E1_2$ and $E1_3$, respectively. These are alternatively spliced onto a common non-coding exon that precedes the exon containing the translation initiation codon. Three alternative first exons are utilized in a tissue-specific manner. $E1_1$ is predominantly present in the ovary, $E1_2$ is specific for the liver, and $E1_3$ is transcribed from the common promoter PIII and is the major form in Leydig cells; it is the minor form in the ovary and the liver. Very recently we have demonstrated that PII directs transcription of the PRLR gene in the rat mammary gland, while PI and PII were not detected. Such multiple and tissue-specific promoter utilization may provide a molecular basis for the differential regulation of PRLR expression in normal as well as neoplastic tissues. The orientation of the three alternative first exons has been recently determined by mapping of several overlapping genomic clones, PIII was localized upstream of PI and PII. By using genomic FISH (fluorescence *in situ* hybridization) method, the three alternate first exons were colocalized to the same chromosomal locus, as was the PRLR coding region, indicating that the rat PRLR gene is transcribed alternatively from at least three promoter regions. Alternate utilization of exons accounted for the multiple forms of 5' UTRs in the PRLR transcripts. Promoter deletion studies performed in mouse Leydig tumor cells (MLTC) and HepG2 cells have localized the minimal promoter domains of PI, PII, and PIII to regions -700 to -549 (5', flanking the initiation site at -549), -606 to -179 (encompassing the initiation site at -405) and -607 to -181 (encompassing the initiation site at -340), respectively. PI exerts strong promoter activity only in MLTC, PII exerts strong activity only in HepG2, and PIII is operative in both cells types. The discrepancy noted between the tissue mRNA transcripts related to PI in rat *in vivo* and the promoter activity of PI constructs in cultured Leydig cells could be due to species differences (PI is exclusively active in ovarian tissue in the rat and is not present in the testis). The suppression of PI probably does not occur in MLTC-1 cells but is revealed in rat testicular Leydig cells *in vivo*. At present there are no available homologous rat Leydig or ovarian cell lines for further studies of tissue specific differences between *in vitro* and *in vivo*. Subsequent studies will be performed using primary cultures of Leydig and ovarian follicular cells.

B. The androgen pathway: Hormonal regulation of testicular function.

Studies of the regulation of androgen synthesis.

Studies of the regulation of androgen synthesis in steroidogenic cells have focused on both transcriptional and post-transcriptional regulation of the proteins that catalyze these reactions; the P450 17α- that catalyzes the production of DHEA or androstenedione in consecutive hydroxylase and lyase activities, and the 17β-hydroxysteroid dehydrogenase (17β-HSD) that catalyzes the conversion of androstenedione to testosterone. Studies of the regulation of the CYP17 lyase activity at the molecular level have utilized species and tissue specific differences to identify target regulatory sequences. Adenovirus infection of CYP17 promoter/luciferase reporter gene constructs, in primary cultures of rat adrenal and rat Leydig cells, revealed a rat-specific domain between -1 and -180 bp that caused lack of both basal and cAMP-induced CYP17 transcription in the adrenal, but not the Leydig cell. In contrast, similar promoter constructs from other species (human and bovine) exhibited substantial cAMP induced transcriptional activity in the rat adrenal. Functional studies indicate that the presence of the rat CRE/ATF domain or the absence of the bovine/human SF-1 domain at this aligned position is not responsible for silencing rat gene transcription in the rat adrenal. This domain has been noted to include an additional SF-1 like element that in our constructs overlaps with the rat SF-1 element. These studies suggest that a mechanism for the differential rat promoter CY17 activity in the Leydig and adrenal cells may involve a specific transcriptional factor that binds to the rat gene but not the human gene, and is specific to only one of the cell types, Leydig or adrenal. The rat and human promoters are driven by SF-1 protein, which is present in both steroidogenic cell types, and a non-SF-1 inhibitor (adrenal) or activator (Leydig) trans-factor may be responsible for the tissue-specific promoter activity.

Structural studies based on mutagenesis of the conserved region of the rat and human proteins reveal significant differences in the amino acid domains required for hydroxylase and lyase activities within and between the two species (human/bovine vs rat), consistent with their differential regulation of lyase activity. The conserved domain 334 to 363 contains a sequence that is highly similar to that of the signature sequence in the active site of the short chain dehydrogenase family (YGXSK vs. YVGXSR339) and a prokaryotic member of that family ($3\alpha/20\beta$ HSD) also contains significant similarity to the essential arginines in this domain. Mutagenesis of R339 from the rat CYP17 (as well as R340 of the human CYP17) of the signature sequence abolished both hydroxylase and lyase activities without affecting protein expression levels, indicating that it is of crucial importance to CYP17 activity, and suggesting a structural link between the CYP17 active site and the short chain dehydrogenase steroid binding domain.

To study the mechanism of differential inhibition of lyase activity in the human protein, we have utilized the rat P450 17α-hydroxylase, since the rat, unlike the human, exhibits the unique property of catalyzing both Δ_4 and Δ_5 lyase activities (the human only catalyzes Δ_5 activity). The previous identification in our laboratory of a CYP17 amino acid Arg346 that is essential only for rat CYP17 lyase activity has been an important indication that this amino acid is either an essential 17α-hydroxylated steroid contact point, or is an essential conduit for external stimulation of only the lyase activity by tissue-specific factors. Sequential mutations of the amino acids surrounding Arg 346 in the rat protein suggested that F343 contributed specifically to Δ_4 lyase activity. When it was substituted to the human counterpart isoleucine, Δ_4 lyase activity was substantially but not totally reduced, whereas hydroxylase activity was not changed. To test whether the F343 to I344 substitution was respnsible for the absence of $\Delta 4$ lyase activity in the human CYP17, the I344 of the human clone was mutated to the rat F to determine if the mutant human P450 17α-hydroxylase recovers the $\Delta 4$ lyase activity. 50% of the rat $\Delta 4$ lyase activity was recovered, indicating that this residue inhibits this activity but is not completely responsible for this specific difference. However, this mutation also resulted in a change in hydroxylase activity in the human protein. Further serial and simultaneous mutations of this domain to the rat counterparts confirmed that position 344 to 349 is important for hydroxylase activity in the human protein, and that the rat sequence 344 to 349 cannot substitute. These observations identify different amino acids in the 343 to 363 domain in the rat and human proteins that are selectively important for hydroxylase or lyase activities to varying degrees. Therefore, it is likely that this domain serves as a steroid binding domain, rather than as a general surface conduit for activators.

Significance to Biomedical Research and the Program of the Institute:

A. Gonadal receptors: structure, regulation, and function. Characterization of LH and prolactin receptors and their relationship to membrane coupling systems will assist in understanding the basic biochemical mechanisms which control the reproductive functions of the testis and ovary. Also, further elucidation of the receptor-cyclase interaction will be of general value in understanding the mechanisms of action of protein hormones.

The recently elucidation of functional domains within the promoter and upstream regions of the LH receptor will permit the complete characterization of the transcriptional mechanism of this atypical promoter, their regulation and the elucidation of processes involved in developmental and tissue-specific expression of the receptor, and its up-and down-regulation at different stages of gonadal development. Analysis of the domains involved in ligand interaction should provide the basis for the development of receptor antagonists. This could also identify critical regions susceptible to mutational inactivation in certain forms of primary hypogonadism. In addition these studies contribute with basic information about mode of transcriptional and regulated activity in TATA-less genes.

Insights into the molecular biology of PRLR promoter usage and control could provide the basis for alternative therapeutic strategies in breast cancer, including the potential for organ selectivity. A targeted therapeutic approach is viewed as preferable since PRLR-mediated actions also participate in the control of the immune system, gonads, and liver. In addition, characterization of promoter(s) in the mouse should contribute to the development of promoter-derived targeted knockout transgenic models for studies of the role of PRLR in the development and progression of breast cancer. The rat studies have shown that expression of the PRLR gene in the ovary and in the liver is under dual promoter control. Although a PRLR antagonist was reported to inhibit the growth of cultured breast cancer cell lines, it would be non-selective if used *in vivo* due to the wide distribution of the prolactin receptor therefore it would be advantageous to develop strategies targeted to the specific promoter of the PRLR utilized in the mammary gland, while maintaining the function of promoters elsewhere in the body. Further studies on the definition of promoter control will provide background information for therapeutic promoter directed ablation.

B. The androgen pathway: hormonal regulation of testicular function.

The hormonal control of Leydig cell function and androgen production is an essential component of male sexual development and reproductive capacity. In addition to its significance to the programs of the Endocrinology and Reproduction Research, this project has the potential to provide knowledge about the general actions of peptide and protein hormones upon target cells in tissues other than the gonads.

The findings on the autocrine/paracrine actions of 5HT/CRF/opiates are not only relevant to basic aspects of the local physiological control of testicular function but also provide new insights into the pathogenesis of stress-related disorders of reproductive function, idiopathic infertility and disorders arising from drug addiction and antihypertensive therapy. It should be possible to design therapeutic strategies addressing the two primary alterations that may result from dysfunction of this complex hormonal regulatory system, CRF's direct inhibitory effect on steroidogenesis and its stimulation of opiatergic tone. Both of these actions could lead to disorders of androgen production and alteration of tubule function responsible for some forms of infertility in men. 5HT, the immediate stimulus of testicular CRF secretion, is released centrally and peripherally during stress, and is locally increased in pathological disturbances of testicular function (i.e., orchitis, varicocele). These findings defining the primary participation of 5HT in the secretion of CRF by the Leydig cells provide an important avenue for novel therapeutic approaches for male infertility through the development of antagonist therapy. In studies of CYP17, the physical separation of hydroxylase from lyase activity, and the importance of domain 343-348 for lyase activity in this enzyme, are relevant to the development of inhibitors of androgen formation for treatment of patients with prostate cancer, and for elucidation of the basis of certain forms of male pseudohermaphroditism.

Proposed Course:

A. Gonadal receptors: structure, regulation, and function.

1. Functional analysis of promoter regions of the rat LH receptor gene. We will proceed with further elucidation of specific regions of the LH receptor gene that are responsible for transcriptional initiation and its regulation (including regions relevant to constitutive, hormone-, 2nd messenger-, and growth factor-dependent inducible transcription). Investigation of differences in promoter activity that might account for differential expression of the LH receptor in the ovary and Leydig cell during development. Investigation of transacting factors that are involved in the LH receptor gene regulation. Identification of cis-regulatory elements associated with the rat LH receptor gene which are relevant and for up and down regulation of receptors.

We will extent studies on the characterization of the M1 protein and proceed with analysis of M1 clones recently obtained by expression cloning of a Leydig cell library. After sequencing these clones we will characterize the protein. This will include the identification of functional domains and the sites of interaction with the M1 DNA element and direct or indirect interactions with relevant protein(s) such as SP1 or other proteins that may be involved in basal and activated transcription. In a similar fashion the proteins interacting with SP1 at the $SP1_2$ domain of the rat promoter region will be investigated.

We will initiate studies on the functional characterization of the human LH receptor gene in a similar fashion as was previously performed for the rat receptor. Several length constructs will be expressed in a number of human cell lines. Recent studies have indicated that the LH receptor is expressed in a number of extragonadal human tissues, therefore an initial screening of current human cell lines will be performed prior to adopting a cell line that would be suitable for our studies. Following identification of the promoter domain which presumably resides in areas close to the initiation of transcription site we will mutate the putative 5' atypical TATA boxes and map the regions of transcriptional control.

2. Investigation of regions and specific amino acids of the extracellular domain required for high-affinity binding, for hormonal specificity, and for signal transduction, by means of deletions, construction of chimeras, and mutations. For further characterization of the binding domain, experiments will include deletions and/of additional selective mutagenesis of leucine repeats which are unique for the LHR will be mutated. These structures may contribute to the binding pocket as well as to the specificity of the hormonal signal. For signal transduction, changes will include mutations of the Cys residues within the transmembrane domain as well as point mutations of transmembrane V and VI and of the third intracellular loop and of the N- or C-region of this loop as well as selected areas of the cytoplasmic domain and mutation of individual amino acids within this region. In addition potential regions of interaction between the extracellular binding domain (empty receptor or following occupancy with the transmembrane domain) and the transmembrane domain. Potential phosphorylation sites will be mutated individually.

3. Further elucidation of the role of carbohydrate residues of the LH receptor in the binding of hormone and their participation on the folding and transit of the receptors to the plasma membrane. Specifically the role of N-acetylglucosamine (linked to Asp in the CHO chain) will be investigated. We will attempt to determine the relevant residue(s) within the receptor that interact with this sugar moiety and participate in the folding reaction induced by the sugar residue. Within the established requirement of the sugar residue for folding and or conformational stability of the binding site we will determine whether a putative fucose attached to the sugar residue participate in the folding of the nascent receptor and conformational stability of the mature receptor.

4. Overexpression of LH receptor forms using a recombinant baculovirus expression system. Large scale production of wild type functional and mutant receptor form B with a range of biological activities and subsequent purification will be obtained for studies on the structure/function aspects of the receptor, for analysis of structural requirements for hormonal binding, and ultimately for analyzing the conformation of the binding pocket.

5. Further characterization of mRNA forms of LH and lactogen receptor. Determination of the nuclear rate of transcription of the LH and prolactin receptor during ovarian and testicular homologous (LH receptor) and heterologous (prolactin receptor) by trophic hormones and 2nd messengers. *In vitro* translation of different mRNA forms or LH and prolactin receptors and identification of products by immunoprecipitation with a battery antibodies to receptor peptides.

6. Further definition of prolactin receptor promoter domains/ tissue specificity in the rat. Assuming that multiplicity of promoter/and tissue specificity is similar for the mouse as was found in the rat we will proceed with selective knock out of the individual promoter that selectively direct the expression of the receptor in testis and ovary. We will proceed with studies on the transcriptional regulation of LH and prolactin receptors by homologous and heterologous hormones.

B. The androgen pathway: hormonal regulation of testicular function.

1. We will further extend the analysis of mechanisms responsible for induction of early and late steroidogenic lesions. These will include studies on the regulation of gene expression of Leydig cell cytochrome P-450 17α-hydroxylase, 17-20 desmolase by hCG and estradiol, and nuclear transcription assays. We will determine structural requirements of the P-450$_{17\alpha}$ for enzymatic activities of 17α-hydroxylase/17-20 desmolase, and will isolate the 5' regulatory gene sequences for investigation of regulation of gene function. We will study direct actions of estradiol, androgen or other modulators on P450$_{17\alpha}$ gene regulation. The expression of enzymatic activities of constructs with P450$_{17\alpha}$ cDNA inserts with deletions of the 1st and/or 2nd transmembrane regions, and in some cases containing a signal peptide of secretory preproteins, will be explored in COS-1 cells. Also, we will carry out expression of P450$_{17\alpha}$ cDNA with selective mutations of regions of the putative binding site to explore further the individual domains of this bifunctional enzyme.

2. Regulation of the last step of the steroid biosynthetic pathway is under investigation. Preliminary studies indicate that activation of the enzyme requires ATP and the enzymatic activity is dependent of adequate entry of glucose into the cells. The rat HSD will be cloned by expression cloning, and or hybridization using oligonucleotide probes from the recently cloned human HSD type 3 isoform. The enzyme will be overexpressed and regulation of its activity will be investigated. Also, HSD cDNA constructs wild type and mutants, will be coexpressed in COS1 cells with CYP17 and the activity of the enzyme will be determined. The role of phosphorylation on the activity of the enzyme will be initially investigated putative phosphorylation sites will be mutated and the activity of the enzyme will be determined.

3. The mechanisms of LH-induced receptor regulation and steroidogenic lesions will be analyzed in cultured Leydig cells. More detailed analysis of the appearance and development of receptors and responses through fetal life, and the influence of homologous and heterologous hormones such as prolactin and FSH and GnRH in culture, will be performed. Further investigations of functional compartmentalization of cAMP will be continued with localization and identification of protein phosphorylation substrates during the action of LH, choleragen and forskolin, along with other stimulatory and inhibitory hormones and substances. This is to be correlated with adenylate and guanylate cyclase activities, cAMP production, and steroidogenesis in fetal and adult Leydig cells.

4. Communications between tubule cells and Leydig cells, and myoid and mesenchymal cells with Leydig cells, will also be studied. The receptors and modulatory actions of a number of peptide hormones of known stimulatory and inhibitory actions in other tissues will be analyzed in Leydig, Sertoli, and germinal cells in attempts to understand homologous regulatory mechanisms within cell populations. We will investigate the nature of the serotonin signal transduction mechanism and membrane coupling functions, and will further dissect the mechanism of the gonadotropin signaling pathway affected by CRF. The dual role of protein kinase C as a mediator of CRF, and presumably of serotonin action in Leydig cells, will also be evaluated.

Publications:

Zhang R, Cai, H, Fatima N, Buczko E, Dufau ML. Functional glycosylation sites of the rat luteinizing hormone receptor required for hormone binding. J Biol Chem 270:21722-21728, 1995.

Dufau, ML. The luteinizing hormone receptor. Current Opinion in Endocrinology and Diabetes. Vol. 2, 2:365-374, 1995.

Zhang R., Buczko E, Dufau ML. Cysteine Residues in exons 1 to 6 of the extracellular domain of the luteinizing hormone receptor are required for hormone binding. J Biol Chem 271:5755-5760, 1996.

Hu Z, Zhuang L, Dufau ML. Multiple and tissue-specific control of gonadal and non-gonadal prolactin receptor gene expression. J Biol Chem 271:10242-10246, 1996.

Tsai-Morris C-H, Buczko E, Geng Y, Gamboa-Pinto A, Dufau, ML. The genomic structure of the corticotropin releasing factor receptor. J Biol Chem 271:14519-14525, 1996.

Dufau ML. The luteinizing hormone receptor. in "The Leydig Cell" (Payne A, Russel L, Hardy M, eds.) Cache River Press, Clearwater, FL, p. 333-350, 1996.

Dufau ML, Miyagawa Y, Takada S, Khanum A, Miyagawa H, Buczko E. Regulation of androgen synthesis: The late steroidogenic pathway. Steroids. (In Press) 1996.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00184-18 ERRB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Pituitary Hormone Secretion

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K.J. Catt	Head, SHR	ERRB, NICHD
Other:	K. Arora	Sr. Staff Fellow	ERRB, NICHD
	L. Krsmanovic	Visiting Scientist	ERRB, NICHD
	Z. Cheng	NRSA Fellow	ERRB, NICHD
	N. Mores	Adjunct Scientist	ERRB, NICHD
	S. Xiao	Adjunct Scientist	ERRB, NICHD

COOPERATING UNITS *(if any)*

Philipps Universitat Marburg, Marburg, Germany (O. Ortmann)

LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Section on Hormonal Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.0	6.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project includes studies on the structural and signaling properties of receptors for gonadotropin-releasing hormone (GnRH), and their role in neuropeptide release and gonadotropin secretion. GnRH receptors were found to be expressed not only in pituitary gonadotrophs but also in the hypothalamus and in immortalized GnRH neurons (GT1 cells). Cultured hypothalamic cells and GT1 cells exhibit pulsatile GnRH release that is modulated by neurotransmitters and neuropeptides including GnRH itself. In structure-function studies on the GnRH receptor, a conserved leucine residue in the second intracellular loop (leu147) was found to be essential for the coupling of the receptor to G protein-mediated signaling responses and for agonist-induced internalization of the ligand-receptor complex. In addition, the aromatic moiety of the conserved tyrosine residue in the atypical NPXXY (DLPIY) sequence of the GnRH receptor was found to be essential for normal coupling to G proteins, and is a critical element in agonist-induced receptor activation and signal transduction. Studies in GT1 cells revealed the presence of large-conductance potassium channels that assist in membrane repolarization but do not participate in the control of neuropeptide release. In both GT1 cells and hypothalamic neurons, nanomolar concentrations of hCG were found to rapidly inhibit the pulsatile release of GnRH in a reversible manner. This process was not mediated by the stimulatory action of hCG on cAMP production, but probably results from a Gi-mediated inhibitory action on plasma-membrane ion channel activity. This effect of hCG or pulsatile GnRH release could be responsible for the suppression of pituitary gonadotropin secretion during pregnancy. Studies of the control of ovarian function by members of the VIP/GHRH/PACAP family of neuropeptides showed that both PACAP and the PACAP-I receptor and expressed in the rat ovary. The presence of PACAP and three splice variants of its type I receptor suggest that PACAP can exert autocrine or paracrine actions on granulosa cell function. In human ovarian cancer cells, endothelin I (ET-1) was found to be produced and to act on ETA receptors to stimulate calcium signaling and thymidine incorporation. The effect of ET-1 on cell proliferation, and the basal growth rate of the tumor cells, were inhibited by the ETA receptor antagonist, BQ123. Thus, ET-1 produced in ovarian cancer cells can act through ETA receptors as an autocrine growth factor, and may contribute to cell growth and proliferation in certain ovarian tumors.

Project Description

Objectives:

To clarify the mechanisms of action of hypothalamic neurohormones and other regulatory factors that control secretion in gonadotropin-releasing hormone (GnRH) neurons and anterior pituitary cells. In particular, to characterize the receptors, signal transduction pathways, and cellular processes involved in the neuronal control of GnRH biosynthesis and secretion, and the effects of GnRH on gonadotropin release. This includes studies on the GnRH-producing neurons of the hypothalamus, immortalized GnRH-secreting neurons, cultured pituitary cells, and αT3-1 gonadotroph cell lines to elucidate the receptor-mediated pathways that control the functions of these cell types.

Methods Employed:

Dispersion and culture of hypothalamic and pituitary cells, and αT3-1 and GT1-7 cell lines. Radioligand assays, solubilization, and photoaffinity labeling of GnRH receptors in pituitary cells and membrane-rich fractions. Column perfusion of cultured pituitary cells and hypothalamic neurons. Radioimmunoassay of cyclic nucleotides, gonadotropins, prolactin, and other hormones in pituitary cells and culture medium. Fractionations of pituitary cell suspensions by density gradient and centrifugal elutriation methods. Measurements of inositol phospholipid turnover and arachidonic acid metabolites in gonadotrophs labeled with radioactive precursors. Assay for diacylglycerol, and of protein kinase C and its distribution in ligand-stimulated cells. Measurements of cytoplasmic calcium concentrations in single cells by photometry and imaging techniques, and in suspension by spectrofluorimetry. Cloning and mutational analysis of GnRH receptors; analysis of pathways and transcription factors regulating GnRH receptor gene expression.

Major Findings:

Structure-function properties of the GnRH receptor. The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), acts via its specific receptors in the anterior pituitary gland to regulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone, and thus plays a pivotal role in reproduction. The cDNAs for the GnRH receptors of several species, including mouse, rat, sheep, cow, and human, have recently been cloned. The deduced amino acid sequences of the receptor share more than 85% amino acid identity among species. The coding region of the GnRH receptor contains seven putative transmembrane domains (TM I-VII) as well as many of the conserved residues and sequences typical of other members of the G protein-coupled receptor (GPCR) superfamily. However, the GnRH receptor exhibits several unique features that include (a) the presence of a long and highly basic first intracellular loop; (b) the replacement of Tyr by Ser in the conserved GPCR "signature" DRY motif located at the junction of TM III and the second intracellular loop; and (c) the absence of a cytoplasmic COOH-terminal tail. Another interesting feature of the receptor is that the highly conserved Asp in TM II and Asn in TM VII are reciprocally exchanged. Upon agonist binding, the GnRH receptor activates phosphoinositide hydrolysis to diacylglycerol and inositol 1,4,5-trisphosphate, thereby mobilizing Ca^{2+} and activating protein kinase C to initiate a variety of cellular responses. Receptor activation is followed by extensive internalization and down-regulation of the receptor via endocytosis and processing. The molecular mechanism(s) of GnRH receptor activation are beginning to be clarified by structure-function studies employing site-directed mitogenesis to identify residues that are involved in G protein activation, signal transduction, and receptor internalization. In the muscarinic receptor, a conserved leucine residue in the second intracellular loop has been implicated in both signal transduction and receptor sequestration. This hydrophobic amino acid is also conserved (as Leu^{147}) in the GnRH receptor and is present at the equivalent position in the 2i loop. In studies on the structure-function relationships of the GnRH receptor, we examined the roles of the conserved Leu^{147} residue and the unique Ser^{140} residue, which is Tyr or Trp in most other GPCRs, in agonist-induced signal transduction and receptor internalization. Replacement of Leu^{147} with either Asp or Ala impaired both G protein coupling and receptor internalization. However, replacement of Ser^{140} by Try did not affect the signaling response but increased ligand binding and receptor internalization.

In the GnRH receptor, the $NPX_{2-3}Y$ motif that is present in the seventh transmembrane helix of most G protein-coupled receptors is unusual in containing Asp instead of Asn but retains the highly conserved Tyr residue. The importance of this aromatic residue in the DPLIY sequence of the GnRH receptor function was analyzed by replacing Tyr^{322} with Ala or Phe residues. The $Y^{322}A$ mutant receptor expressed in COS-7 cells had high agonist binding affinity, but its ability to interact with G protein(s) and to activate inositol phosphate production in response to GnRH was abolished. Although functionally inactive, the $Y^{322}A$ mutant receptor was internalized at about 50% of the rate of the wild type receptor in agonist-treated cells. When Tyr^{322} was replaced with Phe to preserve its aromatic nature, the $Y^{322}F$ mutant receptor displayed normal G protein activation and inositol phosphate responses to GnRH and was internalized in the same manner as the wild type receptor. These findings demonstrate that the aromatic moiety of the Tyr^{322} component of the DPLIY motif in the GnRH receptor is a critical determinant of agonist-induced receptor activation and signal transduction.

Regulation of Ca^{2+} signaling and GnRH release by neurotransmitters.

Neurotransmitters and neuropeptides modulate the secretion of GnRH from the hypothalamus and thus influence the release of gonadotrophic hormones into the circulation. Analysis of the electrophysiological effects of these regulatory factors on GnRH neurons is complicated by the small number and wide distribution of GnRH neurons in the hypothalamus, and the consequent difficulties in isolating and identifying them. The GT1-7 line of immortalized GnRH neurons has many similarities to native GnRH neurons, and has become a useful model in which to investigate the electrophysiology of the GnRH neuron. Recent evidence from GT1-7 cells suggests that the neurotransmitters, γ-aminobutyric acid (GABA) and glutamate, as well as the neuropeptides, endothelin and GnRH, increase GnRH secretion at least in part by depolarizing the plasma membrane, which results in Ca^{2+} influx through L-type Ca^{2+} channels and increased cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). The neuropeptides also increase $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from internal stores.

Calcium-activated potassium [K(Ca)] channels may be involved in the dampening of excitation of GT1-7 neurons following neurotransmitter- and neuropeptide-evoked increases in GnRH release. In order for the GT1-7 cell to return quickly to a low resting $[Ca^{2+}]_i$ following stimulation, the cell must undergo membrane repolarization toward its resting potential of -40 to -70 mV, followed by the closure of its voltage-sensitive Ca^{2+} channels. Delayed rectifier K^+ channels, which open in response to membrane depolarization, may mediate membrane repolarization since the outward K^+ current through the delayed rectifier K^+ channel would drive the cell toward the equilibrium potential for K^+. K(Ca) channels, which open in response to both membrane depolarization and elevated $[Ca^{2+}]_i$ in other types, can aid in this repolarization. GT1 cells have been reported to express a variety of K^+ channels, including a delayed rectifier, an inward rectifier, a 4-aminopyridine (4-AP)-sensitive channel, and a 4-AP and tetraethylammonium (TEA)-insensitive channel. However, whether they also express K(Ca) channels has not been determined. In other cells, two types of K(Ca) channels have been characterized: the large-conductance, K(Ca), or BK, channel and the small-conductance, K(Ca), or SK, channel.

The expression and function of large-conductance Ca^{2+}-activated K^+ (BK) channels in GT1-7 neurons was investigated by measuring ionic currents using the patch-clamp technique. Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored using the flurorescent indicator, fura-2, and GnRH secretion was measured by radioimmunoassay. In cell-attached and inside-out patch-clamp recordings, K^+ channels with a single-channel conductance of ~200 pS were detected. Depolarizing the patch increased the unitary current size and the open probability. In perforated-patch recordings, depolarizing pulses (50 ms) to potentials of -10 to +60 mV from a holding potential of -90 mV elicited outward current with early transient and sustained components. The transient current peaked 2-10 ms after the beginning of each pulse and increased in a voltage-dependent manner. This current was unaffected by the small-conductance Ca^{2+}-activated K^+ channel blocker apamin (100 nM), reduced by the BK channel blocker, charybdotoxin (5 nM), abolished by the Ca^{2+} channel blocker, $CdC1_2$ (25 μM), and prolonged by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (1 μM). Based on the single-channel and whole-cell conductances, the number of channels per patch, the patch area, and the surface area of the cell, each GT1-7 cell contains 30-60 BK channels. The functional role of BK channels in GT1-7 cells was evaluated by measuring the effect of charybdotoxin (100 nM) on basal $[Ca^{2+}]_i$ and GnRH secretion, as well as on the $[Ca^{2+}]_i$ and GnRH secretory responses to γ-aminobutyric acid (GABA, 100 μM), an excitatory neurotransmitter in this system. Charybdotoxin had no effect on basal $[Ca^{2+}]_i$ or GnRH secretion, or on the GABA-evoked $[Ca^{2+}]_i$ or GnRH secretion, or on the GABA-evoked $[Ca^{2+}]_i$ and GnRH secretory responses. These results indicate that GT1-7 cells express BK channels and that the functional role of such channels is not related to the control of GnRH release.

Regulation of pulsatile GnRH release by LH receptors expressed in GnRH neurons.

The hypothalamic GnRH neuronal network releases GnRH in a pulsatile manner to regulate gonadotropin release from the pituitary gland, and is consequently responsible for the control of steroid hormone secretion and gamete production by the gonads. The operation of a short feedback loop between the pituitary gland and the hypothalamus has been indicated by the ability of LH to modulate its own secretion *in vivo*,and to decrease single cell firing rates in the medial preoptic area and basal hypothalamus. In addition, marked inhibition of multiunit electrical activity was observed during the initiation of the preovulatory surge of LH, coincident with the late follicular rise in serum estradiol concentration. Recent studies in cultured hypothalamic neurons, as well as immortalized pituitary gonadotrophs and GnRH-producing neurons, have begun to address the direct involvement of neuropeptides, neurotransmitters, and pituitary hormones in the control of hypothalamic GnRH secretion. Cultured hypothalamic neurons and immortalized GnRH neurons (GT1-7 cells) exhibit pulsatile GnRH release and express plasma-membrane receptors for GnRH, prolactin, and LH, as well as several other hormones and transmitters. In GT1-7 cells, agonist-induced activation of GnRH receptors leads to stimulation of phospholipase C activity, increased formation of Ins(1,4,5)P_3 mobilization of Ca^{2+} from intracellular stores and Ca^{2+} entry through VSCC, and modulation of GnRH release.

Treatment of GT1-7 cells with hCG caused a dose-dependent increase in cAMP production, with a rapid increase during the first 15 min and a subsequent decrease that was prevented by pre-treatment with pertussis toxin. Furthermore, the stimulatory effect of cholera toxin on cAMP production was inhibited by hCG in a dose-dependent manner. These data indicate that the LH receptors expressed in GT1-7 cells are coupled to both adenylyl cyclase stimulatory (G_s) and inhibitory (G_i) proteins. In perifused cell cultures, treatment with forskolin and 8-bromo cAMP increased the amplitude of spontaneous GnRH release. However, treatment with nanomolar concentrations of hCG abolished pulsatile GnRH release from both GT1-7 cells and rat hypothalamic cells. The similarity of hCG action on pulsatile GnRH release to that of extracellular Ca^{2+} depletion and calcium channel antagonists, and its partial resistance to potassium-induced depolarization, suggest that it results from inhibition of plasma-membrane ion channel activity. It is probable that the inhibitory action of hCG on pulsatile GnRH release is responsible for initiation of the suppression of pituitary LH secretion during pregnancy.

Structure of the rat GnRH receptor gene.

The structure of the rat gonadotropin-releasing hormone receptor gene was determined by screening a rat testis genomic library with the cDNA for the mouse GnRH receptor. The gene was found to consist of three exons that encode the receptor protein, and to span about 20 kb of DNA. One genomic clone contained the 5'-untranslated region and the first exon, and the other contained the second and third exons. Thus, the intron-exon structure of the rat receptor gene is similar to those of the mouse and human genes, with intervening sequences located in the coding region at the sites of the fourth transmembrane domain and the third intracellular loop. A putative TATA box is positioned 126 nt upstream of the start codon and 23 nt in front of the transcription initiation site. The 1.7 kb promoter sequence contains an AP-1 site, CCAAT sequences, and a Pit-1 binding site. The sequences of the promoter, exons, and introns of the rat GnRH receptor gene are approximately 85% identical with the corresponding regions of the mouse receptor gene. Current studies are directed at the analysis of the molecular mechanisms underlying transcriptional regulation of the receptor gene, employing luciferase receptor constructs based on selected regions of the 1.8 kb 5'-flanking region of the gene.

Expression of PAPAP receptors in the mammalian ovary.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a hypothalamic neuropeptide that belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP)/growth hormone releasing hormone (GHRH) family of peptide hormones. Two biologically active forms of the PACAP molecule are derived from a single precursor protein of 176 amino acids. The larger form (PACAP-38) contains 38 amino acid residues and the smaller (PACAP-27) consists of its N-terminal 27 amino acid residues and shares 70% identity with VIP. PACAP exerts a potent stimulatory action on cyclic AMP production in anterior pituitary cells, and promotes the release of several pituitary hormones. It also stimulates phosphatidylinositol hydrolysis and increases cytosolic Ca^{2+} in several cell types. Like many other hypothalamic neuropeptides, PACAP and its receptors are present in a variety of extrahypothalamic tissues, including brain, testis, pituitary, respiratory system, and gastrointestinal tract. Furthermore, PACAP stimulates intracellular signaling responses and induces process outgrowth in human neuroblastoma cell lines.

PACAP is known to recognize two major groups of receptor sites. The type I sites have high affinity for PACAP-38 and PACAP-27 and low affinity for VIP. They are further subdivided into type IA (with equal affinity for PACAP-38 and PACAP-27) and type IB (with 100-1000 times higher affinity for PACAP-38 than for PACAP-27). In contrast, the type II sites bind PACAP 38, PACAP-27, and VIP with equal affinity. The cDNA sequence of the rat PACAP-I-R encodes a 467 amino acid protein containing the seven putative transmembrane domains that are characteristic of guanine nucleotide binding protein-coupled receptors. Five splice variants of the PACAP-I-R, with or without insertions at the C-terminal end of the third intracellular loop, have been described. These include hip, a 28 amino acid insert; *hop1* a different 28 amino acid insert; *hop2*, an insert similar to *hop1*, but lacking a single serine residue; and *hip-hop1*, a combination of the *hip* and *hop1* inserts. the PACAP-I-R and its isoforms are highly expressed in many areas of CNS and to a moderate extent in the pituitary gland, adrenal medulla, and testis. The ratio of PACAP-I-R to PACAP-I-*hop1*-R varies between tissues, suggesting that splicing of the PACAP-I-R gene could be differentially regulated in a tissue-specific manner.

Previous studies have demonstrated that VIP and GHRH, peptides that are structurally related to PACAP, exert biological actions on ovarian function by stimulating granulosa cells to produce cAMP, progesterone, and estrogen. PACAP has also been found to stimulate steroidogenesis and cAMP accumulation in cultured rat granulosa cells. Immunoreactive PACAP is present throughout the human female genital tract, and has been detected at low levels in the rat ovary. Expression of the PACAP gene has been observed in the catfish ovary, but has not been reported in the mammalian ovary. The present study was performed to analyze the expression of PACAP and its receptors in the rat ovary, where peptides of the PACAP/VIP/GHRH family act as local regulators of granulosa cell function.

In the rat ovary, expression of the PACAP gene was demonstrated by amplification of ovarian RNA by the reverse transcription/polymerase chain reaction (RT-PCR). In addition, hybridization of Northern blots of rat ovarian poly$(A)^+$ RNA with a 706-nt rat hypothalamic PACAP-I receptor cDNA probe revealed the presence of a 7.0 kb PACAP receptor transcript, similar to that detected in brain and hypothalamus. RT-PCR using specific primers for the PACAP-I-R gene yielded products of the expected size with RNA obtained from ovarian tissue, brain, and hypothalamus. The authenticity of the PCR products was confirmed by Southern blotting and nested PCR, which revealed at least three splice variants of the PACAP-I-R in the rat ovary. These findings demonstrate that both PACAP and PACAP-I receptor isoforms are expressed in the rat ovary, where they could exert autocrine and/or paracrine actions on granulosa cell function.

Autocrine actions of endothelin in ovarian cancer cells.

The identification of ET-1 as a potent vasoconstrictor peptide produced by vascular endothelial cells was followed by the recognition of a family of 21-amino acid isopeptides, termed ET-1, ET-2, and ET-3. Endothelins exert their biological actions through at least two major receptor subtypes, ET_A and ET_B, that are defined by their relative affinities for ET-1, ET-2, and ET-3. ET_A receptors are characterized by high and equal affinity for ET-1 and ET-2, and 100-fold lower affinity for ET-3, whereas ET_B receptors display high and similar affinity for all three endothelin peptides. ETs and sarafotoxins initiate a common signal transduction pathway, principally through G protein-mediated activation of phospholipase C and subsequent inositol trisphosphate-dependent increases in intracellular Ca^{2+} levels.

ET peptides and their receptors are expressed in numerous tissues and cell types, including the cardiovascular and nervous systems, and appear to be involved in a wide variety of physiological functions. In addition to activating phosphoinositide hydrolysis and calcium signaling, ET-1 stimulates mitogenic and proliferative responses in vascular smooth muscle and other cell types, as well as the rapid expression of *c-fos* and *c-myc*. These and other findings have demonstrated that ET-1, like several other calcium-mobilizing hormones, has the capacity to elicit growth responses in its target tissues. Recently, ET-1 has been found to be produced in a number of neoplasms, including laryngeal, lung, hepatocellular, pancreatic, colon, mammary, and cervical carcinomas. In conjunction with observations that ET-1 has TGF-β-like activity on the anchorage-independent growth of NRK 49F cells, its production by a variety of tumor cells has led to the proposal that ET-1 may participate in the pathogenesis of certain types of cancer through an autocrine/paracrine mechanism. Recent studies on ovarian cancer, which is a major cause of death from gynecological malignancy, have focused on factors that regulate the growth of malignant ovarian epithelium. A more fundamental understanding of these factors might suggest novel and potentially more effective approaches to the treatment of ovarian neoplasms. Significant amounts of ET-1 mRNA are present in the bovine ovary, and immunoreactive ET-1 has been detected in culture medium of porcine granulosa cells and in ovarian follicular fluid. However, little is known about the production and actions of ET-1 in ovarian carcinoma cells.

The production of ET-1 and its receptor-mediated actions on calcium signaling and growth responses were analyzed in human ovarian carcinoma cells. Immunoreactive ET-1 was released from three of four ovarian tumor cell lines as a function of time in amounts ranging from 56 to 74 fmol/10^6 cells. Reverse-phase HPLC and radioimmunoassay of conditioned media from tumor cells revealed a single peak coeluting with authentic ET-1. Radioligand binding studies showed that the ET-1 producing cell lines also expressed high-affinity ET_A receptors (K_d<0.1 nM) that ranged in abundance from 2,600 to 43,600 sites/cell. In fura-2-loaded ovarian carcinoma cells, ET-1 induced dose-dependent increases in cytoplasmic Ca^{2+} concentration. ET-1 also stimulated thymidine incorporation in the three cell lines that expressed ET receptors. In OVCA 433 cells, BQ 123 inhibited the stimulatory actions of ET-1 on thymidine incorporation and cell proliferation, and substantially reduced the basal growth rate of unstimulated ovarian tumor cells. These results demonstrate that ET-1 is produced in ovarian cancer cells and acts as an autocrine growth factor on ETA receptors to stimulate calcium signaling and proliferative responses. Such findings suggest that ET-1 participates in the progression of neoplastic growth in certain ovarian tumors.

Significance of Biomedical Research and the Program of the Institute:

The clarification of receptor-mediated mechanisms by which neurotransmitters and other regulators control GnRH release, as well as the effects of GnRH on the development and function of gonadotrophs is essential for understanding of normal and disordered hypothalamo-pituitary functions. The regulation of gonadotropin secretion is of central importance in reproductive endocrinology, and the roles of GnRH, steroid hormones and other factors in this process, and their respective biochemical mechanisms of action, need to be more fully elucidated. The effects of GnRH on reduction of its receptor mRNA levels implicates the participation of an ultra-short feedback effect of GnRH on receptor synthesis that is of potential importance for use in fertility regulation and suppression of pituitary function. The expression of GABA and glutamate receptors in GnRH neurons, and their coupling to calcium signaling and GnRH release, provides a useful model for further investigation of this type of neuronal receptors in the control of neuroendocrine function.

Proposed Course:

Further studies on the signaling and secretory profiles of normal hypothalamic neurons and GT1 immortalized neurons will be pursed in experiments with several peptide agonists and neurotransmitters. Specific attention will be given to the characterization of an intrapituitary GnRH system, as well as to interactions between hypothalamic and pituitary GnRH systems and the kinetic aspects of the receptor activation and desensitization during the integrative actions of these two systems. Also, characterization of the inhibitory factors involved in control of GnRH release, including the possible effects of the pituitary hormones, LH and prolactin, on signaling and secretion by GnRH neurons are planned. The characterization of endothelin receptors in hypothalamic cells, and the role of endothelin as a regulator of GnRH and gonadotropin release, will be further analyzed. The structure-function properties of the GnRH receptor gene and the GnRH receptor will also be further investigated, including mutational analysis to determine the regions involved in ligand coupling to G protein(s) and internalization. Experiments with permeabilized gonadotrophs and αT3-1 cells will be performed to introduce specific antibodies against the intracellular domains of GnRH receptors. Permeabilization studies will also be explored in investigation of the molecular mechanism of exocytosis in gonadotrophs and GnRH neurons, with specific emphasis on the functions of protein kinase C. In this regard, characterization of the isozymes of protein kinase C in pituitary cells, αT3-1 gonadotrophs, and GT1 neurons will be done, as well as the translocation of those enzymes in agonist-stimulated cells. Also, activation of MAP kinase by GnRH, and the coordinate actions of MAP kinase and protein kinase C in cellular functions will be investigated.

Publications:

1. Arora KK, Sakai A, Catt KJ. Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. J Biol Chem, 1995;270:22820-22826.

2. Arora KK, Cheng Z, Catt KJ. Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor. Mol Endocrinol 1996, 10:979-986.

3. Bagnato A, Tecce R, Moretti C, DiCastro V, Spergel D, Catt KJ. Autocrine actions of endothelin-1 as a growth factor in human ovarian carcinoma cells. Clin Cancer Res 1995;1:1059-1066.

4. Cesnjaj M, Zheng L, Catt KJ, Stojilkovic SS. Dependence of stimulus-transcription coupling on phospholipase D in agonist-stimulated pituitary cells. Mol Biol Cell 1995;6:1037-1047.

5. Mores N, Krsmanovic LZ, Catt KJ. Activation of LH receptors expressed in GnRH neurons stimulates cyclic AMP production and inhibits pulsatile neuropeptide release. Endocrinology (in press).

6. Krsmanovic LZ, Stojilkovic SS, Catt KJ. Pulsatile gonadotropin-releasing hormone release and its regulation. Trends Endocrinol Metab 1995; 7:56-59.

7. Scaldaferri L, Arora K, Lee SH, Catt KJ, Moretti C. Expression of PACAP and its type-I receptor isoforms in the rat ovary. Mol Cell Endocrinol 1996;117:227-232, 1996.

8. Spergel, DJ, Catt, KJ, Rojas E. Immortalized GnRH neurons express large-conductance calcium-activated potassium channels. Neuroendocrinology 1996;63:101-111.

9. Stojilkovic SS, Catt KJ. Novel aspects of GnRH-induced intracellular signaling and secretion in pituitary gonadotrophs. J Neuroendocrinol 1995;7:739-757.

10. Stojilkovic SS, Catt KJ. Receptors and secretory actions of endothelins in neuroendocrine cells. Front Endocrinol 1995;15:135-144.

11. Stojilkovic SS, Zheng L, Tomic M, Cesnjaj M, Jobin RM, Catt KJ. GnRH-induced intracellular signaling in pituitary gonadotrophs. The Second Internal Symposium on Fertility Regulation 1995 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01HD00187-17 ERRB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Hormonal Regulation of Cellular Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K.-P. Huang	Head, SMR	ERRB, NICHD
Others:	F. Huang	Staff Scientist	ERRB, NICHD
	J. Li	Visiting Fellow	ERRB, NICHD
	M. Uh	Visiting Fellow	ERRB, NICHD
	J. Pak	Visiting Fellow	ERRB, NICHD

COOPERATING UNITS *(if any)*

Lab of Mammalian Genes and Development, NICHD, NIH (H. Westphal); Lab of Biochemical Pharmacology, NIDDK, NIH (P. McPhie); Department de Biologie Moleculaire et Structurale, INSERM Unite 244, Grenoble, France (J. Baudier).

LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Section on Metabolic Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Covalent modifications of protein by phosphorylation and oxidation are important mechanisms for the modulation of numerous cellular functions. Protein phosphorylation by protein kinase C (PKC) has been linked to the regulation of a plethora of cellular events. A physiological target of PKC, neurogranin (Ng), is also modified by nitric oxide and other oxidants. Ng binds calmodulin (CaM) with high affinity in the presence of low levels of calcium. Rat bovine, chicken, and human Ng have identical PKC phosphorylation site/CaM-binding domains. Phosphorylation by PKC reduces their binding affinity for CaM and thus favors activation of CaM-dependent enzymes. Rat brain Ng contains four cysteines located at positions 3, 4, 9, and 51. Site-directed mutagenesis showed Cys9 and Cys51 to be essential for the oxidant-mediated intramolecular disulfide bridging that attenuates the CaM-binding affinity of Ng. Bovine Ng and a human variant (Ng-2) lack Cys51, and chicken Ng and another human variant (Ng-1), lack both Cys9 and Cys51. Based on these structural variations we predict that the oxidant-mediated regulation of Ng function, unlike that by PKC, is variable among species. A novel 28 kDa PKC/CK2 substrate, termed HASPP28, has been identified and its cDNA cloned. This protein is a substrate of CK2 in N1E115 neuroblastoma cells, and its phosphorylation and protein level are cell cycle-regulated. Genomic clones containing > 90% of the coding region and 1.2 kb upstream of the transcription start site, without intron sequences, have been characterized. Promoter activity was defined with nested deletion constructs fused to a luciferase reporter gene; EGF, dBu-cAMP, and PMA caused 2-3 fold stimulation whereas PDGF, bFGF, and insulin had no effect. Immunoprecipitated HASPP28 contains a CK2-like kinase that phosphorylates endogenous HASPP28 or exogenous recombinant protein. The HASPP28-associated protein is being investigated by yeast two-hybrid system. The functional roles of the various PKC isozymes in the control of cellular differentiation were analyzed in N1E115 neuroblastoma cells. PKC-activating phorbol ester counteracts the cAMP-induced neurite outgrowth (NOG) of these cells by selective translocation and downregulation of PKC alpha and delta, whereas PKC beta and zeta are not greatly affected. Stable transfectants carrying antisense PKC alpha or delta, with reduced levels of the respective PKCs, were more sensitive to dBu-cAMP for NOG. These two antisense PKC stable transfectants were also less responsive to PMA in counteracting cAMP-induced NOG, suggesting that PKC alpha and delta are involved in regulating NOG.

Project Description

Objectives:

To investigate the hormonal regulation of cellular functions in normal and disordered target tissues. Effort is focused on the delineation of the signal transduction mechanisms in different tissues involving protein kinase- and redox-regulated cellular functions. Current studies are directed to investigate the role of PKC in mediating the actions of hormones and neurotransmitters; to identify novel PKC substrates in the brain; to determine the signaling mechanism of nitric oxide in CNS; to determine the developmental control of PKC and neurogranin gene expressions in neural tissues; to characterize the physiological functions of a novel cell cycle-regulated phosphoprotein, HASPP28; to investigate the regulation of HASPP28 gene expression; and to determine the roles of PKC isozymes in cellular differentiation.

Methods Employed:

This project utilizes a variety of chemical, biochemical, and immunological techniques to study the hormonal regulation of metabolic processes at the molecular and cellular levels. Various enzymes were purified from mammalian tissues and enzyme activities were assayed by spectrophotometric or radiometric methods. Purified enzymes were characterized by their molecular weights as determined by gel electrophoresis, gel filtration, and density gradient centrifugation, by their kinetic properties, and by their substrate specificities. The effects of protein phosphorylation and oxidation were defined by the changes in the activity related parameters and the sites of modification. The phosphorylation sites, defined by the characteristic tryptic peptides, were identified by peptide mapping on thin-layer plate, by isoelectric focusing on polyacrylamide gel, by high-performance liquid chromatography, and by amino acids sequence analysis. Antibodies were prepared by immunization with purified protein or synthetic peptide and purified by affinity chromatography. Immunological characterization of the antigen was carried out by immunoblotting and by light and electron microscopy. Identification of specific mRNA was carried out by Northern blot analysis using DNA probes obtained by molecular cloning. Characterization of specific gene product was done by transfection of cells and followed by biochemical and immunological identification of expressed protein. Recombinant proteins were produced in bacteria. Genomic structure was analyzed by molecular cloning and DNA sequence analysis. Identification of specific DNA-binding protein was carried out by gel-retardation and footprinting analyses.

Major Findings:

Oxidation of rat brain neurogranin by nitric oxide. Neurogranin (Ng) is a 78-amino acid postsynaptic protein kinase C (PKC) substrate present in the neurons of the adult brain. This protein is a high affinity calmodulin (CaM)-binding protein at low levels of Ca^{2+}. Phosphorylation of Ng by PKC at a single site located within a conserved 19-amino acid Ng/neuromodulin homology region, which also contains the predicted CaM-binding domain, results in a weakening of its binding affinity for CaM. It has been hypothesized that the PKC-catalyzed phosphorylation of Ng and neuromodulin frees the CaM for other CaM-dependent enzymes. We have shown that rat brain Ng, which contains four cysteine residues, was readily oxidized by nitric oxide donors, DEANO and sodium nitroprusside, to generate a large conformational change that could be detected by non-reducing SDS-polyacrylamide gel electrophoresis. The reduced Ng had an apparent M_r = 17k and the oxidized one M_r = 10k; reversible conversion between these two M_r species were seen by treatment of Ng with these oxidants and followed by reduction with sulfhydryl reagent. Both the non-phosphorylated and PKC-phosphorylated Ng were susceptible to the oxidation-induced changes in M_r; modification of all four cysteine residues in Ng with 4-vinylpyridine prevented the oxidation-induced changes. Modification of Ng by DEANO was inhibited by calmodulin (CaM) without Ca^{2+} and in the presence of Ca^{2+}, CaM could not protect Ng from oxidation by DEANO. CaM had no protective effect on DEANO-mediated oxidation of the PKC-phosphorylated Ng with or without Ca^{2+}. Oxidation of Ng by the various oxidants resulted in the formation of intramolecular disulfide bond that exhibited lower M_r on SDS-PAGE; this oxidized form displayed lower affinity for CaM than the reduced form as evidenced by CaM-affinity column chromatography. Comparison of the rate of oxidation of Ng by DEANO with other sulfhydryl reagents, it was evident that Ng ranked as one of the best nitric oxide acceptors among those tested including dithiothreitol, glutathione, and serum albumin. Ng associated with rat brain synaptosomes can also be oxidized by DEANO when determined by immunoblot with a specific antibody. Ng in the rat brain extracts is readily oxidized in the presence of Ca^{2+} and the oxidized Ng can be reduced by NADPH, suggesting that there are enzymes catalyzing the interconversion of Ng oxidation/reduction. This study suggests that Ng is a likely target of nitric oxide, in addition to PKC, and that modification of Ng by nitric oxide and other oxidants could serve as a signaling mechanism for controlling the level of CaM, which is essential for many Ca^{2+}-regulated cellular processes.

Site-directed mutagenesis of rat brain neurogranin to determine the cysteine residues involved in intramolecular disulfide bond formation. Rat brain Ng contains four cysteine residues, $Cys^{3,4,9,51}$, all of which are likely targets of nitric oxide. Modification of Ng by nitric oxide is apparently mediated by the formation of a nitroso intermediate followed by disulfide bond formation. Constructs containing single, double, and tetra mutants of the four cysteine residues of rat brain Ng were generated for examining the role of individual cysteine residues in forming intramolecular disulfide bond. The mutant proteins were produced in *E. coli* and purified to near homogeneity. Mutation of all four Cys residues or single mutation of Cys^{51} blocked the oxidant-mediated intramolecular disulfide formation as monitored by the downward mobility shift under non-reducing SDS-PAGE. Single mutation of Cys^3, Cys^4, or Cys^9 or double mutation of any pair of these three Cys residues did not block such intramolecular disulfide formation, although the rates of oxidation of these mutant proteins were different. Thus, Cys^{51} is an essential pairing partner in NO-mediated intramolecular disulfide formation in Ng. Cys^3, Cys^4, and Cys^9 individually could pair with Cys^{51} and the order of reactivity was $Cys^9 > Cys^4 > Cys^3$, suggesting that Cys^9 and Cys^{51} form the preferential disulfide bridge. In all cases tested, the intramolecularly disulfide bridged Ng proteins displayed dramatically attenuated CaM-binding affinity and ~2-3-fold weaker PKC substrate phosphorylation activity. The data indicate that the N-terminal Cys^3, Cys^4, and Cys^9 are in close proximity to the C-terminal Cys^{51} in solution. Disulfide bridge between the N- and C-terminal domains of Ng renders the central CaM-binding and phosphorylation site domain in a fixed conformation unfavorable for binding to CaM and as a substrate of PKC. The Cys51 mutant is similar to the bovine Ng and a human Ng-2 variant, which are predicted not to form intramolecular disulfide as the rat Ng.

Cloning of bovine, chicken, and human neurogranin cDNAs. Rat brain Ng contains four Cys residues, among them Cys^9 and Cys^{51} are crucial for the nitric oxide-mediated intramolecular disulfide bond formation. We investigated if Ng from other sources functioned similarly as rat brain protein following oxidation by nitric oxide. PCR cloning of bovine Ng cDNA revealed a single amino acid substitution of the rat Ng Cys^{51} with Arg^{51}. The bovine Ng was expressed in *E coli* and purified to near homogeneity. As expected, the bovine Ng does not form intramolecular disulfide bond as was observed for the rat brain Ng. Upon exposure to air in the absence of reducing agent, the bovine Ng formed polymers as a result of intermolecular disulfide bond formation. The cloned chicken Ng does not contain Cys^9 nor Cys^{51} , these two amino acids are substituted with Ser^9 and Gly^{51}, respectively. The *E coli* expressed chicken Ng is present predominantly as monomer. Two human Ng cDNAs have been cloned from commercial cDNAs derived from pooled human brain mRNA of several deceased individuals. Neither human Ng-1 nor -2 clones contains Cys^{51} . The human Ng-1 contains Gly^{51} and Ser^9 and Ng-2 contains Arg^{51} and Cys^9 ; the former is similar to the chicken Ng and the latter to the bovine Ng. The *E coli* expressed human Ng-1 and Ng-2 behaved similarly as the chicken and bovine Ng, respectively, upon oxidation. Comparison of the amino acid sequences among rat, bovine, chicken, and two human clones indicates that the variations among the various Ngs are at positions 9 and 51. Single point mutation of rat Ng codon 9 from TGC to AGC converts Cys to Ser for both chicken and human Ng-1 and single point mutation of rat codon 51 from TGT to CGT converts Cys to Arg for bovine Ng and to GGT converts Cys to Gly for both chicken and human Ng-1. Single point mutation of bovine Ng codon 51 from CGT to CGC for human Ng-2 retains Arg^{51} for both clones. The PKC phosphorylation site and the CaM-binding domain of all the Ng clones from different sources are completely conserved. These findings suggest that Ngs from different sources are similarly regulated by PKC; however, they display different responses to the oxidant-mediated signaling.

Generation of neurogranin-deficient mice. A mouse genomic library was screened by plaque hybridization with a ^{32}P-randomly labeled full length Ng cDNA probes. Two independent positive clones were identified from 2x10^6 recombinant plaques. Restriction mapping and Southern analysis of the purified phage DNA indicated clone D331 contained ~11kb insert covering 5'-flanking region and first exon and clone D411 contained ~9kb insert covering 5'-flanking region, first exon, first intron, and 54 bp of second exon. DNA sequence of mouse clone D411 shows high degree of identity to the rat Ng genomic clone. A targeting vector is being constructed in which the altered mouse Ng gene is inserted into a plasmid containing bacterial LacZ, neomycin-resistance, and herpes simplex virus thymidine kinase genes.

Characterization of the promoter activity of the 5' flanking region of HASPP28 gene. HASPP28 is a ubiquitous phosphoprotein, whose cDNA was recently cloned. Though *in vitro*, this protein can be phosphorylated by several kinases including PKA, PKC, and CK1 and 2, in intact cells, its phosphorylation seems to be catalyzed by a CK2-like kinase. Furthermore, phosphorylation of HASPP28 as well as its protein expression level appear to be regulated through cell cycle progression. In order to understand better of the mode of regulation of its expression and phosphorylation, we cloned the HASPP28 gene and characterized its 5'-flanking region. Two genomic fragments, dl3r7 (+509 to -189) and dl5rl (+82 to -1231), have been identified by PCR cloning employed a "Promoter Finder DNA Walking Kit". The dl3r7 genomic fragment covering more than 90% of the coding region without containing interrupting sequence of intron. It appears that HASPP28 is encoded by an intronless gene. Primer extension experiments had identified multiple transcription start sites and -55nt being the most distal one from the translation start site, comparable to the previously cloned cDNA which contains 28 bp in the 5' untranslated region. Computer analysis of the entire 5' flanking sequence of dl5rl revealed numerous putative transcription regulation elements both common and unique. Most notably, there are three TATA boxes and three TFIID element sequences including one of each within 150 bp proximal to the transcription initiation site. This property uniquely qualify this promoter as highly regulatable especially by RNA pol II, and this gene is by no mean a "housekeeping" gene. Furthermore, many transcription regulatory elements appear in pairs within the 1.2 kb 5' flanking region; some are in the same orientation and mostly are in the antiparallel orientation, suggesting the dimerization action pattern of the transactivation binding proteins. A successive series of deletion constructs upstream from the translation start site were fused with a luciferase-carrying reporter plasmid pGL2 basic, namely pGL2-3 (-1231/14), pGL2-4 (-1021/-4), pGL2-5 (-773/-4), pGL2-6 (-582/-4), pGL2-7 (-382/-4), pGL2-1 (-189/-4), and pGL2-8 (-773/-347). These plasmids were transfected into COS-7 cells to characterize the promoter activities exhibited by the 5' flanking sequences. When normalized against cotransfected β-gal activity and compared to the promoterless pGL2 basic plasmid, pGL2-4, -5, and -6 showed at least 10-15 fold activation in the luciferase activity expression, while pGL2-3 had 8-10 fold. On the other hand, pGL2-7, -1, and -8 showed only 3-4, 2-3, and 1.5-3 fold activation, respectively. These results indicate that the basal elements required for the transcription reside within -582nt to -382nt upstream from the translation start site, as activity downstream from -382nt was drastically reduced. Plasmid pGL2-3, though had the longest sequence, did not exhibit the strongest promoter activity, suggesting the presence of a suppressor element between -1231 and -1021. It is surprising that these various constructs exhibit merely 2-5% of the activity of the positive control plasmid of pGL2-SV40 promoter, implying that either a potent activating element is situated still upstream of -1231nt or that the element(s) within this sequence is highly responsive to extracellular stimulus which is not present under the present assay condition. Several potential modulators were tested for their effects on the promoter activities in COS cells. Among these, EGF, dBu-cAMP, and PMA showed 2-3 fold increase in the luciferase activities, while PDGF (AA or BB), bFGF and insulin only affected insignificantly. Though, there is a unique ATF element for CREB (tgacgat) situated at -1101nt which is beyond pGL2-4, and a unique TRE/AP1 site (gtgactca) situated at -170nt which is deleted in pGL2-8, all the plasmids constructs are effectively stimulated by both dBu-cAMP and PMA.

Identification of HASPP28 interacting protein by the yeast two-hybrid system. HASPP28 is a physiological substrate of CK2, however, the biological role of this protein has not been elucidated. In intact cells, HASPP28 is associated with a CK2-like kinase, as the kinase can be coimmunoprecipitated and phosphorylates the associated HASPP28 or exogenously added recombinant protein. Attempt to identify the coprecipitated kinase with CK2 antibodies, however, was not successful. As CK2 itself is a CDK-like kinase, it is possible that

HASPP28 could be a subunit of the associated CK2-like kinase, analogous to the CDK system. In order to gain insight into the biological function of HASPP28, it is necessary to identify the protein(s) it interacts with. The *in vivo* two-hybrid system of yeast seems to be one of the best approaches to determine the HASPP28-associated proteins. This system is based on the modular nature of the eukaryotic transcriptional activator complex GAL4, which consisted two domains namely the DNA binding domain (BD) and the transcriptional activating domain (AD). The bait protein (such as HASPP28) can be fused with BD in the vector and cotransfected with vector containing fusion of unknown interacting protein and AD into special yeast host capable of reporting a successful valid interacting BD and AD. At the present, we have successfully constructed the fusion of HASPP28 and BD into pAS2-1 vector (Trp^+) and cotransfected with pGAD10 (Leu^+), a rat brain library of AD fusions, into LacZ reporter host of yeast CG-1945 (His^+). At least three rounds of selections are necessary to confirm a positive clone expressing a HASPP28 interacting protein. Any successful selection will enable us to amplify and determine the DNA sequence of the protein. In the mean time, we also plan to isolate HASPP28 biochemically from rat tissue under nondenaturing condition hoping to capture HASPP28 with its partner protein associated with it.

Effect of PMA and antisense-inhibition of PKC in the cAMP-mediated NOG of N1E115 neuroblastoma cells. Neurite outgrowth (NOG) in N1E115 cell is induced by dBu-cAMP and inhibited by PMA treatment. Previous results indicate that PMA treatment rapidly translocates and downregulates PKC α and δ in these cells, slowly translocates and only slightly downregulates PKC β1, while leaving PKC ξ unaffected. In PMA prolonged-treated cells, where PKC α and δ are completely downregulated, further PMA treatment no longer inhibits dBu-cAMP-mediated NOG. It was postulated that either PKC α or δ or both were responsible in the inhibition of cAMP-mediated NOG. In order to differentiate the role of each individual PKC and to eliminate the secondary effect resulted from prolonged PMA treatment, we set about to develop stable transfectants where either PKC α or δ are downregulated by transfection with pCDNA3 vector containing G418 selection marker carrying antisense DNA of PKC α or δ, respectively, covering mostly the 5' untranslated region. Several NIE 115/ASα and /ASδ clones were selected and established, and they showed, as analyzed by immunoblotting, various degree of reduction in the level of PKC α and δ. Clones with stable elevated PKC α expression through transfection carrying full length sense sequence were also established. Morphological examination indicated that both representative clones of ASα and ASδ showed higher sensitivities toward dBu-cAMP in terms of NOG in the present or absence of PMA, while elevated PKC α rendered these cells more resistant toward cAMP effect in the same experiment. Following prolonged PMA treatment, again PMA no longer exerted any hindrance toward cAMP-mediated NOG. Interestingly, PMA normally would retract already elaborated neurite from prolonged cAMP treatment, however, in cells which had reduced level of PKC α or δ, the retraction of neuritis did not occurred readily. These observations demonstrate that both PKC α and δ are likely involved in the inhibition of cAMP-mediated NOG in these cells.

Significance to Biochemical Research and the Program of the Institute:

It is the aim of this project to investigate the mechanisms by which hormones, neurotransmitters, and growth factors regulate cellular functions. It is generally believed that the ligand-receptor interaction triggers the release of second messenger(s) which affect(s) the activities of key enzymes through covalent modification of proteins. The signaling systems using cAMP, Ca^{2+}/CaM, and Ca $^{2+}$/diacylglycerol frequently cause changes in the state of phosphorylation and that using nitric oxide causes changes in the state of oxidation of proteins. All these signaling pathways regulate every aspect of living processes including growth, differentiation, metabolism, sensory transduction, immune response, and learning and memory. Understanding the mechanisms of action of these second messengers in controlling the various cellular responses will lead to the development of effective therapy for the related diseases.

Stimulation of the Ca^{2+}/diacylglycerol/PKC signaling pathway lead to the phosphorylation of numerous target substrates. A group of CaM-binding PKC substrates, including neurogranin (Ng), neuromodulin, and MARCKS, have been shown to be physiological targets of the kinase. Phosphorylation of these proteins by PKC favors the release of CaM which becomes available for many CaM-dependent enzymes. This signal amplification step is essential to transduce the extracellular signals for the pleiotropic cellular responses. In addition to the phosphorylation-mediated regulation of its CaM-binding affinity, we have identified Ng as a potential target of nitric oxide and other oxidants. One of the most intriguing findings of our study is the discovery of the oxidation/reduction-mediated regulation of Ng and CaM interaction. Oxidation of rat brain Ng by nitric oxide or hydrogen peroxide forms intramolecular disulfide bond resulting in gross conformational changes and thus reducing the affinity for CaM. The oxidized Ng can be reduced by sulfhydryl reagent to regenerate the high affinity CaM-binding protein. As CaM is an essential component for the regulation of numerous enzymes, including several CaM-dependent protein kinases, adenylyl cyclase, nitric oxide synthase, Ca^{2+} pump, protein phosphatase, cAMP phosphodiesterase, and IP_3 kinase, this newly discovered signaling mechanism involving reversible oxidation/reduction to control the affinity for CaM could be involved in many physiological responses. In the CNS, the excitatory amino acid-mediated stimulation of NMDA receptor that leads to the generation of nitric oxide could increase the free CaM level in the target cells. This mechanism functions in concert with the excitatory amino acid-mediated increase in $[Ca^{2+}]_i$, turnover of membrane phospholipids generating lipid second messengers (diacylglycerol), and activation of PKC that enhances the phosphorylation of Ng. Since an increase in $[Ca^{2+}]_i$, generation of nitric oxide, and activation of PKC have been linked to numerous neuronal functions including enhancement of neuronal plasticity, it is likely that the release of CaM from the CaM-binding PKC substrates may participate in the learning and memory as demonstrated in the long-term potentiation model. Molecular cloning of Ng cDNAs from different sources indicates that the PKC-mediated regulation of Ng from rat, bovine, chicken, and human is highly conserved; however, the oxidation-mediated regulation is variable due to substitutions of the crucial Cys residues with other amino acids. Detailed study of the regulatory mechanisms of the various Ng variants, which is still in progress, will provide clues to the functional evolution of Ng in the neurons.

In order to further define the mechanism by which PKCs transduce the external signal, we investigated the presence of other potential target substrates of PKC in the brain. As a first step for systematic analysis of these proteins, we purified several acid-stable proteins as potential substrates of PKC. Amino acid sequence analysis of these proteins revealed several novel PKC substrates. This group of perchloric acid-soluble proteins contained prominent PKC substrates, neurogranin, neuromodulin, and MARCKS, as well as many nuclear proteins, such as histones and high-mobility group chromosomal proteins. We have cloned the cDNA of a novel M_r=28k phosphoprotein having a broad tissue distribution. This protein, named HASPP28, has been shown to be an *in vivo* substrate of CK2 and both the state of phosphorylation and protein level are cell cycle-regulated. The discovery and cloning of this novel PKC/CK2 substrate will lead to a new research area related to the PKC/CK2-mediated signaling pathway. The role of PKC isozymes in controlling cellular differentiation was carried out with the antisense technology. By selective suppression of a PKC isozyme in the transfected cells expressing the corresponding antisense DNA construct, we have defined the roles of PKC α and δ in the cAMP-induced neurite outgrowth model of N1E115 cells.

Proposed Course:

This project represents an integrated and comprehensive approach to understand the control of cellular functions by reversible covalent modification of proteins. Both the phosphorylation and oxidation are the major focus of this study. Because PKC and its down-stream target substrates have been implicated in the signal transduction to regulate many physiological processes, we will continue to focus our efforts on studying the physicochemical properties and physiological functions of the various PKC substrates; identification of novel PKC substrates by protein purification and molecular cloning; and characterization of the regulatory mechanism for the expression of PKC and its substrates; characterization of the physiological functions of Ng and HASPP28; and to define the role of PKC isozymes in neurite outgrowth.

The discovery of Ng as a redox-sensitive CaM-binding protein raises many interesting questions concerning whether this regulatory process occurs *in vivo*. An antibody against rat brain Ng has been prepared and will be used to detect the nitric oxide-induced modification of this protein. Because of the ease of detection of Ng oxidation by SDS-PAGE and immunoblot, this system will become very useful for characterizing the nitric oxide signaling mechanism in CNS. Since both the phosphorylation and oxidation of Ng reduce its binding affinity to CaM, it is likely that the excitatory amino acid-induced activation of PKC and production of nitric oxide may affect the cellular responses through modifications of Ng. Stimulation of neurons by excitatory amino acids that induces long-term potentiation or neurotoxicity can be monitored by following the changes in Ng molecular weight. Effect of nitric oxide in the modification of Ng in intact cells will also be tested in the wild type and mutant Ng cDNA-transfected cell lines. The physiological function of Ng will also be tested in the Ng-deficient mutant mice its gene has been disrupted.

Ng from rat, bovine, chicken, and human and the various rat Ng mutant proteins have been prepared in milligram quantity. These various proteins are different in their responses to oxidants due to mutation at the crucial Cys residues involved in disulfide bond formation. The functional implication of these variations will be investigated in terms of their phosphorylations by PKC and their affinities for CaM. The structure feature of this protein that endowed with potent reducing power will be explored by studying its three dimensional structure by NMR and X-ray crystallography. The reduced, oxidized, phosphorylated, and phosphorylated/oxidized Ng will be used. Further mutagenesis study to define the structural features of Ng as a potent reductant will be investigated. This protein possesses certain structural features of those neurotoxins capable of modulating ion channels. The functional role of this protein will be tested for its ability to modulate ion channels by patch-clamp analysis with whole-cell and single channel patch. Wild type and mutant Ng in reduced, oxidized, PKC-phosphorylated, and phosphorylated/oxidized forms will be tested for their effects on the various cationic and anionic channels. Both phosphorylation/dephosphorylation and oxidation/reduction of Ng influence the binding of Ng to CaM that serves as signal amplification steps of diacylglycerol and nitric oxide second messenger systems. It is of interest to determine the reversal of PKC phosphorylation by protein phosphatase and nitric oxide oxidation by either chemical or enzymatic reduction. Previously, we have shown dephosphorylation of Ng by protein phosphatase 1, 2A, and 2B; it has yet to determine if the phosphorylated/oxidized Ng can be effectively dephosphorylated by these phosphatases. Reduction of the oxidized Ng occurs *in vitro* by DTT; we have yet to determine the reduction potential of Ng oxidation and the effectiveness of biological reductant, such as glutathione, to reduce the oxidized Ng. It is also possible that an enzyme similar to glutathione reductase that utilizes NADPH for reduction of Ng. Oxidation of Ng by nitric oxide generates nitroso-Ng intermediate which may serve as a donor of nitric oxide for other acceptors, such as glutathione and other proteins.

Identification of target substrates of a protein kinase is essential to understand the signaling mechanism of a second messenger system. A novel M_r=28k rat brain PKC substrate, HASPP28, has been purified to near homogeneity and its cDNA cloned. This protein is an *in vivo* CK2 substrate and the phosphorylation and protein level were regulated in a cell cycle-dependent manner. However, the functional role of this protein is unknown. Attempt will be made to determine i) if the association of HASPP28 with CK2-like kinase is cell cycle-dependent and the phosphorylation affects their association; ii) if this specific phosphorylation serves as a signal for downregulation of HASPP28 at mitotic stage; iii) whether the degradation of HASPP28 is carried out through the ubiquitin-proteasome pathway; and iv) a putative receptor protein of HASPP28 in the detergent-insensitive but salt-sensitive particulate fraction of the cells. We are also constructing i) expression plasmids for producing recombinant proteins in bacteria of site-directly mutated HASPP28, namely, S62A, S62C, and S59AS62A, so to demonstrate that phosphorylation of S59, which is not a CK2 site, is a consequence to the phosphorylation of S62; ii) mammalian expression plasmids with HASPP28 (both wild type and mutated ones) fused N-terminally to a green fluorescent protein (GFP) so to follow the whereabouts of the protein during the cell cycle progression and upon cell stimulation. HASPP28 protein contains a highly acidic region within which the two CK2 phosphorylation sites are located. Phosphorylation of these two Ser residues leads to the presence of a stretch of 11 consecutive acidic amino acids. This region may be involved in the phosphorylation-dependent binding of metal ions or basic proteins.

Immunoprecipitation of the salt-extracted HASPP28 contains an associated protein kinase that phosphorylates the protein at the sites corresponding to the *in vitro* CK2 phosphorylation sites. This HASPP28-associated kinase, however, is dissimilar to the authentic CK2 in that it does not phosphorylate the peptide substrate for

CK2. Interactions of HASPP28 with its kinase will be investigated by density gradient centrifugation and gel-filtration chromatography and by yeast two-hybrid system to identify all the possible *in vivo* interacting proteins.

Since HASPP28 is present at its lowest level in the mitotic cells, it is possible that its expression is also dictated by the cell cycle stages. We have already cloned the 5'-flanking region of this gene and characterized the promoter activity with several nested deletion constructs. A more detailed mutational analysis for the promoter activity by both microdeletion and base substitution will be needed to further define the sequence elements important for transcriptional regulation. Several other potential regulators, such as glucocorticoid, estrogen, and keratinocyte enhancer, all of which have defined sites in HASPP28 gene, will be tested for their effects. The roles of TATA/TFIID, TRE/AP1, AP2, and NfκB, all of them situated within 150 nt upstream of the transcription start site, in the control of the proximal promoter activity will be analyzed. Using nuclear extracts or commercially available purified TFIID, AP1 (c-jun), AP2, and NfκB, the DNAase I footprinting analysis will be carried out to identify the DNA sequence protected by these proteins. Synthetic oligonucleotides with base substitutions will be utilized in the gel mobility shift assay and to dissect the mechanism of transcriptional regulation of this gene. The sequence region important for cell cycle stage induction or repression will also be investigated.

In the N1E115 cell differentiation model, prolonged PMA treatment and PKC β1 antisense inhibition downregulate PKC α, β1, and δ to a minimum level, yet PMA inhibition of cAMP-mediated NOG is not completely relieved. It is possible that the remaining PKC activity or a non-PKC but PMA responsive component is responsible for the residual inhibition. There is also the PMA-non-responsive PKC ζ, which is constitutively active and which may block the cAMP effect. Stable transfectants expressing antisense PKC α or δ enhance the response to cAMP for NOG and diminish the antagonistic effect of PMA on cAMP. Thus, PKC α or δ must play pivotal roles in NOG. Other studies have indicated that the initial morphological differentiation (NOG) as affected by cAMP and PMA is a manifestation of cytoskeletal protein rearrangement without requiring new protein synthesis. We plan to characterize types of cytoskeletal proteins, actin, microtubule, or neurofilament, etc. involved and the mechanism of the opposing actions between cAMP/PKA and PMA/PKC in these proteins that causing morphological changes.

Publications:

Li H, Chen H-C, Huang FL. Identification of a rapidly dephosphorylating 95 kDa protein as elongation factor 2 during 8-Br-cAMP treatment of N1E115 neuroblastoma cells. Biochem Biophy Res Comm, 1995;217:131-137.

Mahoney CW, Seki K, Huang K-P. Phosphorylation of MARCKS, neuromodulin, and neurogranin by protein kinase C exhibits differential responses to diacylglycerols. Cellular Signalling, 1995;7:679-685.

Mahoney, CW, Pak J, Huang K-P. Nitric oxide modification of neurogranin: identification of the cysteine residues involved in intramolecular disulfide bridge formation using site-directed mutagenesis. J Biol Chem, 1996; in press.

Seki K, Sheu, F-S, Huang K-P. Binding of myristoylated alanine-rich protein kinase C substrate (MARCKS) to phosphoinositides attenuates the phosphorylation by protein kinase C. Arch Biochem Biophy, 1996;326:193-201.

Shen L, Huang K-P, Chen H-C, Huang FL. Molecular cloning and characterization of a novel casein kinase II substrate, HASPP28, from rat brain. Arch Biochem Biophy, 1996;327:131-141.

Sheu F-S, Mahoney CW, Seki, K, Huang, K-P. Nitric oxide modification of rat brain neurogranin affects both the phosphorylation by protein kinase C and the affinity for calmodulin. J Biol Chem, 1996; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00193-11 ERRB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Angiotensin II Receptors and Activation Mechanisms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K.J. Catt	Head, SHR	ERRB, NICHD
Others:	T. Balla	Visiting Scientist	ERRB, NICHD
	A.J. Baukal	Chemist GS-12	ERRB, NICHD
	H. Ji	IRTA Fellow	ERRB, NICHD
	Y. Tian	Visiting Fellow	ERRB, NICHD
	G. Downing	Adjunct Scientist	ERRB, NICHD
	R. Smith	Adjunct Scientist	ERRB, NICHD

COOPERATING UNITS *(if any)*

Department of Physiology, Semmelweis University Medical School, Budapest (L. Hunyady).
Department of Chemical Endocrinology, Medical College of St. Bartholomew's Hospital, London (AJL Clark)

LAB/BRANCH

Endocrinology and Reproduction Research Branch

SECTION

Section on Hormonal Regulation

INSTITUTE AND LOCATION

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.5	6.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Structure-function studies on the AT1 receptor, which mediates most of the known physiological actions of angiotensin II (Ang II), have revealed further aspects of the domains involved in receptor activation and signal transduction. In addition to a conserved tyrosine residue (Tyr-215) in the 5th transmembrane domain, a conserved apolar amino acid (Leu-222) in the amino-terminal region of the third cytoplasmic loop was found to be essential for the activation of the AT1 receptor by Ang II. The Lys-222 mutant receptor was unable to adopt the high-affinity binding conformation, did not couple to G proteins, and did not mediate activation of phosphoinositide hydrolysis. A search revealed that most G protein-coupled receptors contain an apolar residue in this position, suggesting that this amino acid is an essential element in the agonist-induced activation not only of the AT1 but possibly many other such receptors. The AT1a and AT1b receptor subtypes expressed in stably transfected adrenal Y1 cells exhibited small but significant differences in their binding pharmacology, and were coupled through a pertussis-insensitive Gq-type protein to the phosphoinositide/calcium signaling pathway. However, in contrast to their behavior in several Ang II target tissues, neither subtype was coupled through Gi to inhibition of adenylyl cyclase when expressed in murine adrenal tumor cells or COS cells. An orphan receptor was isolated in the course of cloning the angiotensin AT1b receptor and was subsequently identified as the adrenomedullin receptor, based on its binding pharmacology, tissue distribution, and functional properties. The identification of this receptor cDNA should facilitate investigation of the regulation and actions of adrenomedullin in a wide range of normal and neoplastic tissues. The recently identified cytosolic wortmannin-sensitive phosphatidylinositol 4-kinase (PI4K) was found to be distinct from the recognized membrane-bound type II PI4K, but shared several properties with the membrane-bound Type III PI4K of bovine brain. Thus, Type III rather than type II PI4K enzymes are responsible for the maintenance of the precursor phospholipid pools that are required for agonist-induced signaling through the inositol phosphate/calcium pathway. Studies on agonist-induced calcium transients in adrenal glomerulosa cells revealed that calcium influx through VSCCs modulates the frequency of the oscillatory calcium signals elicited by physiological Ang II concentrations. However, at higher agonist concentrations Ca^{2+} influx occurs predominantly through the capacitative entry pathway, and influx through VSCCs makes little contribution to the resulting non-oscillatory calcium signals.

Project Description

Objectives:

The aims of this project are to characterize the cell-surface receptors for angiotensin II (Ang II) and to define the signal transduction pathways involved in the stimulation of target-cell responses such as activation of aldosterone secretion during Ang II action. Studies include the analysis of radioligand binding properties of membrane-bound and solubilized Ang II receptors; the isolation and characterization of photolabeled Ang II receptors; cloning of cDNAs for mammalian and amphibian Ang II receptors; characterization of endogenous and expressed mammalian Ang II receptors in *Xenopus* oocytes and transfected cells; mutational analysis of Ang II receptors to identify amino acids involved in ligand binding, receptor activation and G protein coupling, and endocytosis of agonist-receptor complexes; investigation of the mechanisms and role of calcium entry and mobilization during Ang II action; analysis of the changes in phospholipid turnover and inositol polyphosphate metabolism that mediate the cellular actions of Ang II in adrenal glomerulosa and other angiotensin target cells; and investigation of the signaling pathways through which Ang II controls growth and proliferation of adrenal glomerulosa cells.

Methods Employed:

Radioligand binding studies with Ang II agonist, antagonist, and photoreactive derivatives; solubilization and fractionation of free and radiolabeled receptor sites; fluorescence analysis of cytoplasmic calcium levels in cell suspensions and single cells; measurement of calcium release from intracellular stores and subcellular fractions; HPLC and TLC analysis of phosphoinositides and inositol phosphates; radioligand studies of intracellular $InsP_3$ receptors; radioimmunoassays of aldosterone, pregnenolone and cyclic nucleotides; expression of adrenal and other mRNAs in *Xenopus* oocytes; Northern, Southern and Western blots; construction of expression libraries; polymerase chain reaction; molecular cloning and sequencing of Ang II receptor subtypes; transient and stable transfection of mammalian cells with receptor cDNAs.

Major Findings:

Structure-function studies on AT_1 receptors: Since the cloning of the Ang II receptors in 1992, efforts have been made to identify structural features, specifically amino acid residues and sequences, that are involved in the processes of ligand binding, agonist activation and G protein coupling, and internalization of agonist-receptor complexes. However, much remains to be discovered about the individual residues involved in these processes, and the intramolecular interactions between specific regions of the receptor that lead to the conformational changes necessary to adopt the active conformation of the receptor. This problem applies not only to the Ang II receptors, but to all members of the G protein-coupled (GPCR) superfamily. We have previously defined the residues that form the binding site for the non-peptide antagonist, losartan, which are located within the transmembrane domains of the receptor. In addition, residues within the cytoplasmic tail of the receptor that are essential for agonist-induced internalization oy the ligand-receptor complex have been identified. Current studies have focused on the identification of residues that are involved in the Ang II-induced activation of the receptor, and its coupling to the plasma-membrane effector system that mediate intracellular signaling responses.

Receptor activation and signal transduction. The AT_1 receptor has been reported to interact with several G proteins, including G_q, G_i, and G_o, but its major physiological functions are expressed through G_q-mediated activation of phospholipase C followed by phosphoinositide hydrolysis and Ca^{2+} signaling. Although all GPCRs possess the basic seven-transmembrane structure, only a few amino acids highly conserved among the superfamily of G protein-coupled receptors. In most GPCRs, the binding site for physiological agonists is formed by amino acids located in superficial regions of the intramembrane helices and the extracellular regions of the receptor. The conformational change evoked by agonist binding is transmitted by the transmembrane helices to the intracellular loops, which are generally believed to couple the receptor to its cognate G protein(s) and thence to intracellular signaling systems. Mutagenesis and biochemical studies have demonstrated that the amino-terminal portion of the third intracellular loop is a major structural element involved in receptor activation and G protein recognition. Secondary structure predictions suggest that this region is an amphiphilic α-helical extension of the fifth transmembrane helix. In accordance with the proposed importance of this region in G protein activation, synthetic peptides corresponding to the N-terminal portion of the third intracellular loop can activate the appropriate G proteins *in vitro*. However, relatively few studies have identified specific amino acids that are required for receptor activation, and the structural determinants of the G protein coupling specificities of GPCRs are still unclear.

Site-directed mutagenesis studies with muscarinic, β-adrenergic, formyl peptide, and Ang II receptors have found no evidence that charged residues in this amphiphilic segment participate in G protein coupling. However, uncharged amino acids in this portion of the third intracellular loop may be important determinants of G protein recognition and activation. One such residue is Tyr^{254} in the M_3 muscarinic receptor, which is conserved between muscarinic receptors that activate phosphoinositide signaling (M_1, M_3 and M_5) but is replaced by a serine residue in those (M_2 and M_4) that inhibit adenylyl cyclase. However, most of the GPCRs that stimulate phosphoinositide turnover do not possess a tyrosine residue in this position. Identification of other amino acids that determine G protein coupling is important in providing clues about the nature of the interaction between the receptor and its G proteins(s), and the mechanisms of G protein activation and specificity.

Previous deletion studies and a recent report utilizing chimeric AT_1/AT_2 receptors have identified the amino-terminal portion of the third intracellular loop as a critical region for G protein coupling of the rat AT_{1a} receptor. We also observed that substitution of phenylalanine for a conserved tyrosine residue (Tyr^{215}) in the fifth transmembrane domain, near the origin of the third cytoplasmic loop, caused loss of AT_{1a} receptor activation. The coupling of agonist-activated seven transmembrane domain receptors to G proteins is known to involve the amino-terminal region of their third cytoplasmic loop. Analysis of the amino acids in this region of the AT_1 receptor identified Leu^{222} as an essential residue in receptor activation by Ang II. Non-polar replacements for Leu^{222} yielded functionally intact AT_1 receptors, while polar or charged residues caused progressive impairment of inositol phosphate generation without affecting receptor affinity for the peptide antagonist, [Sar^1,Ile^8]Ang II. The decrease in Ang II-induce signal generation was associated with a parallel reduction of receptor internalization, and was most pronounced for the Lys^{222} mutant receptor. Although this mutant showed normal antagonist binding, its affinity for Ang II was markedly reduced, indicating that the Lys^{222} receptor is unable to adopt the high affinity conformation. A search revealed that many G_q-coupled receptors contain an apolar amino acid (frequently leucine) in the position corresponding to Leu^{222} of the AT_{1a} receptor. These findings demonstrate that this conserved residue in the third intracellular loop is a key element in the agonist-induced activation of the AT_{1a} receptor.

Properties of AT_1 receptor subtypes expressed in adrenal Y1 cells.

Angiotensin II receptors are among the most widely distributed receptors and regulate the functions of numerous target cells. Their most important physiological role is to mediate the effects of Ang II in the control of blood pressure by influencing vascular tone and regulating extracellular fluid volume through effects on salt and water balance. Studies on the pharmacology and functional properties of Ang II receptors in various tissues and cells have revealed significant heterogeneity of the receptors in terms of their size, second messenger systems, and sensitivity to reducing agents. Recently, the use of novel peptide and nonpeptide antagonists has demonstrated the existence of two major subtypes of the Ang II receptor, AT_1 and AT_2, and has clarified some of the earlier differences observed between Ang II receptors in certain tissues. Most of the known regulatory functions of Ang II in its target cells have been found to be mediated by AT_1 receptors. However, signaling processes regulated by AT_2 receptors, including regulation of ion channels and phosphotyrosine phosphatase activity, have been recently identified.

The cloning of AT_1 receptors from vascular smooth muscle and bovine adrenal cells has more completely defined the molecular basis of Ang II receptor heterogeneity. Two closely related forms of the AT_1 receptor have been identified in the rat and mouse. These exhibit marked DNA sequence homology in their coding regions but differ significantly in their noncoding regions and in their relative tissue distributions. These AT_1 receptor subtypes, termed AT_{1a} and AT_{1b}, are present in most Ang II-responsive tissues in varying proportions, which complicates the analysis of their binding pharmacology and coupling to intracellular effector systems. Studies on the properties of the two AT_1 receptor subtypes expressed in COS-7 cells and in *Xenopus laevis* oocytes have suggested differences in their ligand selectivity and regulation of Ca^{2+} signaling. The present report describes a more complete characterization of the rat AT_{1a} and AT_{1b} receptor subtypes stably expressed in adrenocortical Y-1 tumor cells.

Adrenocortical Y-1 cells were stably transfected with the AT_{1a} and AT_{1b} subtypes of the rat AT_1 receptor cDNA to study the pharmacological and functional properties of the two receptors. Selected clones of transfected cells expressing the AT_{1a} or AT_{1b} receptor subtypes bound the native ligand Ang II and the peptide antagonist [Sar^1,Ile^8]Ang II with similar affinities, but they differed in their relative affinities for the nonpeptide antagonist losartan (IC_{50} 9.7 and 4.7 nM), Ang III (126 and 33 nM), and the peptide antagonist [Sar^1,Gly^8]Ang II (6.2 and 1.2 nM). Photoaffinity labeling of the expressed receptors revealed a single component of 65 kDa for both receptor subtypes, suggesting that both receptors were glycosylated in a similar manner. The sensitivity of ^{125}I-Ang II binding to AT_{1a} and AT_{1b} receptors to guanine nucleotides was unaffected by pertussis toxin treatment. Ang II stimulated the formation of inositol phosphates and increased the level of cytoplasmic Ca^{2+} in both AT_{1a}- and AT_{1b}-transfected Y-1 cells. However, Ang II had little effect on forskolin-induced adenosine 3', 5'-cyclic monophosphate accumulation, causing only minor inhibition in AT_{1a}-and AT_{1b}-transfected Y-1 cells. These data indicate that AT_{1a} and AT_{1b} receptors show small but significant differences in their binding pharmacology and, upon activation, are coupled through G_q/G_{11} to the phosphoinositide-Ca^{2+} signaling pathway. However, neither AT_{1a} nor AT_{1b} receptors exhibit coupling to G_i and inhibition of adenylate cyclase when expressed in murine adrenal tumor cells.

A novel phosphatidylinositol 4-kinase essential for agonist-induced Ca^{2+} signaling.

We recently observed that the fungal metabolite, wortmannin (WT), a potent inhibitor of myosin light chain kinase, prevented the sustained but not the initial increases in Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ in Ang II-stimulated adrenal glomerulosa cells. The inhibition of Ca^{2+} influx by WT was found to be a consequence of reduced Ins(1,4,5)P_3 formation and could reflect the resultant refilling of the agonist-sensitive Ca^{2+} pool. Further exploration of the site of action of WT focused on the pathway responsible for biosynthesis and maintenance of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] the immediate precursor of intracellular signals generated by calcium-mobilizing hormones and growth factors. The synthesis of PtdIns(4,5)P_2 is initiated by the conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate [PtdIns(4)P] by phosphatidylinositol 4-kinase (PtdIns 4-kinase). Although cells contain several PtdIns 4-kinases, the enzyme responsible for regulating the synthesis of hormone-sensitive PtdIns(4,5)P_2 pools has not been identified.

Micromolar concentration of WT were found to inhibit the synthesis of hormone-sensitive PtdIns(4)P and PtdIns(4,5)P_2 pools in intact adrenal glomerulosa cells, and a WT-sensitive PtdIns 4-kinase was identified in adrenocortical extracts. In addition to its sensitivity to the PtdIns 3-kinase inhibitor WT, this enzyme is distinguished from the recognized membrane-bound PtdIns 4-kinases by its molecular size and weak membrane association. Inhibition of this PtdIns 4-kinase by WT results in rapid loss of the hormone-sensitive PtdIns(4,5)P_2 pool in angiotensin II-stimulated glomerulosa cells. Consequently, WT treatment inhibits the sustained but not the initial increases in inositol 1,4,5-trisphosphate and cytoplasmic $[Ca^{2+}]$ in a variety of agonist-stimulated cells, including adrenal glomerulosa cells, NIH 3T3 fibroblasts, and Jurkat lymphoblasts. These results indicate that a specific WT-sensitive PtdIns 4-kinase is critical for the maintenance of the agonist-sensitive polyphosphoinositide pool in several cell types.

While the role of PtdIns(4,5)P_2 in intracellular signaling is well recognized, it has become evident that polyphosphoinosides are also involved in the regulation of more diverse cellular functions. Actin binding proteins such as profilin have been shown to bind PtdIns(4,5)P_2, and this interaction has a significant impact on the ability of phospholipase C isozymes to hydrolyze the lipid. Adaptor proteins that participate in clathrin-coated pit assembly (and hence receptor-mediated endocytosis) also bind PtdIns(4,5)P_2 (as well as some highly phosphorylated inositol derivatives), and such interactions affect their function. A separate nuclear phosphoinositide system is regulated by as yet unknown mechanisms that are distinct from those involved in the classical plasma membrane phosphoinositide system. More recently, the critical role of PtdIns(4,5)P_2 in the G nucleotide-dependent activation and association of phospholipase D with various small G proteins (ARFs and Rho) has been the focus of numerous studies.

In contrast to these multiple functions of PtdIns(4,5)P_2, the manner in which 3-phosphorylated inositol phospholipids influence growth responses and other important cell functions has yet to be defined. The 3-phosphorylated inositides represent only a small fraction of the total phosphoinositide population and are produced by PtdIns 3-kinases. These enzymes are activated by receptor tyrosine kinases as well as by oncogenic tyrosine kinases, and are important for several aspects of cell regulation. Since most of the known actions of phosphoinositides require the phosphorylation of PtdIns, the phospholipid kinases are essential participants in the regulation of multiple cell functions. Although the importance of 4-phosphorylated inositides was recognized much earlier than that of the 3-phosphorylated forms, considerably more is known about PtdIns 3-kinases than about PtdIns 4-kinase. The first PtdIns 3-kinase to be isolated and cloned has 110 kDa catalytic and 85 kDa regulatory subunits, and phosphorylates PtdIns and PtdIns-(4))P as well as PtdIns(4,5)P_2. Additional 3-kinases have been described recently, including a PtdIns-specific 3-kinase that phosphorylates only PtdIns but not its phosphorylated derivatives, and others that are regulated by G protein $\beta\gamma$ subunits. These enzymes are soluble and can be inhibited by wortmannin, a fungal metabolite, with potencies that vary among the individual forms of the enzyme.

Although receptor-regulated formation of the calcium-mobilizing second messenger, Ins(1,4,5)P_3, is highly dependent on the formation of PtdIns(4,5)P_2 by PtdIns 4-kinase and PtdIns(4)P 5-kinase, the PtdIns 4-kinase involved in this pathway has not yet been identified. PtdIns 4-kinase activity is most abundant in the membrane fraction of homogenized tissues and can be solubilized by detergents, so most attempts to purify the enzyme have utilized the membrane-bound forms. In the brain, two forms of detergent-soluble PtdIns 4-kinase have been distinguished: type II, a smaller (~56 kDa) adenosine-sensitive enzyme; and type III, a larger (>200 kDa) form that is less sensitive to inhibition by adenosine. In addition, we have identified a cytosolic PtdIns 4-kinase (cPI4K) in the bovine adrenal cortex. This novel enzyme is sensitive to the PtdIns 3-kinase inhibitor, wortmannin, and is essential for the maintenance of agonist-sensitive PtdIns(4,5)P_2 pools in several cell types. Our recent studies have focused on the characteristics of this soluble kinase, and the comparison of its properties with those of the membrane-bound type II adrenal PtdIns 4-kinase and the brain type III enzyme.

The enzymatic activity of adrenal cPI4K was inhibited not only by WT (IC_{50}~ 50 nM) but also by LY-294002 (IC_{50} ~ 100 μM), another inhibitor of PtdIns 3-kinase, and neither compound affected type II PtdIns 4-kinase at concentrations that inhibited cPI4K. In contrast to the type II enzyme, cPI4K had a significantly higher K_m for ATP, was relatively insensitive to inhibition by adenosine (K_i ~ 800 μM vs 40 μM), had lower affinity for PtdIns, and was not inhibited by Ca^{2+} ions. These properties identify the WT-sensitive adrenal cPI4K as a type III PtdIns 4-kinase that is distinct from the tightly membrane-bound, Ca^{2+}- and adenosine-sensitive, type II PtdIns 4-kinase. The type III PtdIns 4-kinase prepared from bovine brain exhibited similar kinetic parameters as the adrenal cPI4K, and was also inhibited by WT with an IC_{50} of 30-50 nM. Since WT inhibits the synthesis of agonist-regulated phosphoinositide pools in intact cells at micromolar concentrations, these findings indicate that type III rather than type II PtdIns 4-kinases are responsible for the maintenance of the precursor phospholipids required for intracellular signaling through the inositol phosphate/Ca^{2+} pathway.

Calcium signaling in adrenal glomerulosa cells.

Repetitive Ca^{2+} transients occur in a variety of cell types following stimulation with low concentrations of Ca^{2+} mobilizing agonists, and consist of a series of Ca^{2+} spikes that occur at intervals of 0.5 to 5 min, depending on the agonist dose and the cell type. Such oscillatory signals often persist for 1 hour or more, and have been observed in cell types ranging from electrically non-excitable cells such as hepatocytes, through partially excitable endocrine and neuroendocrine cells (e.g., pancreatic β cells, pituitary cells, and adrenal glomerulosa cells) to classical excitable cells (muscle cells, neurons). In many cases the spiking frequency, rather than the amplitude of the oscillations, is regulated by the agonist concentration, providing the basis of a frequency-encoded Ca^{2+} signaling mechanism. Although Ca^{2+} release from internal stores is a major source of the cytoplasmic Ca^{2+} transients, $[Ca^{2+}]_i$ levels are also influenced by Ca^{2+} entry mechanisms operating through plasma membrane Ca^{2+} channels. These include a ubiquitous form of Ca^{2+} influx in which the filling state of the Ca^{2+} stores regulates Ca^{2+} influx ("capacitative Ca^{2+} entry"), as well as various types of voltage-sensitive calcium channels (VSCC) that determine Ca^{2+} influx as a function of the membrane potential in excitable cells.

Recent models to explain the generation of Ca^{2+} oscillations have focused on the dual regulation of the Ins(1,4,5)P_3 receptor channel by its ambient cytosolic Ca^{2+} concentrations, with positive and negative feedback by low and high $[Ca^{2+}]_i$, respectively. This mechanism has been proposed to initiate and terminate short bursts of Ca^{2+} release even at constant $InsP_3$ levels. The restoration of basal $[Ca^{2+}]_i$ between spikes depends on diverse Ca^{2+} homeostatic mechanisms, including Ca^{2+} pumps located in the endoplasmic reticulum (ER) and plasma membrane (PM). These processes, and possibly uptake by mitochondria, re-establish the resting $[Ca^{2+}]_i$ levels and also refill the Ca^{2+} pools so that a new cycle can be initiated. Due to the high Ca^{2+} sensitivity of the Ins(1,4,5)P_3 receptor channel, disturbances in the Ca^{2+} homeostatic mechanism(s) of the cell frequently affect the rate and character of Ca^{2+} oscillations. For example, the Ca^{2+} wave frequency in *Xenopus* oocytes can be accelerated by enhancement of Ca^{2+} influx, as well as by overexpression of a SERCA type Ca^{2+} pump to promote the rapid resetting of basal $[Ca^{2+}]_i$ levels. These findings indicate the involvement of both Ca^{2+} entry and reuptake mechanisms in the regulation of periodic calcium signaling in these large cells. Recent microfluorimetric studies revealed that small microdomains, or so-called hot spots, with little impact on the average $[Ca^{2+}]_i$ of the cell may have a crucial role in the regulation of intracellular Ca^{2+} signaling events. This implies that if the Ca^{2+} stores with their Ins(1,4,5)P_3 receptors and the plasma membrane Ca^{2+} entry channels are close to one another, even small amounts of Ca^{2+} entering through these channels could be an important factor in the regulation of Ca^{2+} signals by the local effects of Ca^{2+} influx.

In the adrenal glomerulosa cell, the presence of both capacitative Ca^{2+} entry pathway and various types of VSCC have been reported. Both of these Ca^{2+} influx mechanisms are active during Ang II stimulation of bovine glomerulosa cells, providing two complementary means to regulate intracellular Ca^{2+} signaling through Ca^{2+} entry. Thus, dihydrophyridines have been shown to exert minor but consistent effects on the $[Ca^{2+}]_i$ plateau following stimulation with high doses of Ang II in suspensions of adrenal zona glomerulosa cells. Such results have suggested that voltage-dependent Ca^{2+} channels make relatively little contribution to the Ca^{2+} signals generated by Ang II in adrenal cells, which contrasts with the sensitivity of the secretory response of these cells to dihydropyridines, especially at low Ang II concentrations. This apparent controversy could be resolved if the role of VSCC activation is not to increase the overall cytosolic Ca^{2+} levels but rather to modulate Ca^{2+} oscillations which occur primarily at lower, more physiological doses. The relationships between the Ca^{2+} influx pathways and the Ca^{2+} release process that is the basis of Ca^{2+} oscillations were analyzed in Ang II-stimulated bovine glomerulosa cells. Ang II (100 pM) induced repetitive $[Ca^{2+}]_i$ spikes, the frequency of which was regulated over a narrow range of Ang II concentrations. In the absence of externally added Ca^{2+}, Ca^{2+} oscillations were maintained for prolonged periods of time but their frequency was significantly lower than in the presence of Ca^{2+} (0.23 min^{-1} vs. 0.38 min^{-1}, respectively). Elevation of $[Ca^{2+}]_e$ from zero to 0.6 mM increased the frequency of Ca^{2+} oscillations but only in cells that showed narrow spikes with undiminishing amplitude. However, in cells with broad spikes of markedly decreasing amplitude Ca^{2+} addition changed oscillations to a sustained plateau of $[Ca^{2+}]_i$. In the presence of Ca^{2+}, the frequency of the oscillatory Ca^{2+} response to 100 pM Ang II was reduced (by 49%) by nifedipine and was accelerated (by 86%) by BAY K 8644 or shifted to a sustained $[Ca^{2+}]_i$ plateau normally evoked by higher Ang II concentrations (>1 nM). These findings indicate that Ca^{2+} influx through VSCCs modulates the frequency of synchronized Ca^{2+} oscillations induced by low agonist concentrations by a mechanism that does not involve major changes in inositol trisphosphate formation. In contrast, VSCCs make relatively little contribution to the non-oscillatory Ca^{2+} signals generated by high agonist concentrations, when Ca^{2+} influx occurs primarily through the capacitative Ca^{2+} entry pathway.

Cloning and expression of a mammalian adrenomedullin receptor.

The potent hypotensive peptide, adrenomedullin (AM), was originally isolated from a human adrenal pheochromocytoma using a detection system based on its ability to elevate platelet cAMP levels. Subsequent work revealed that this peptide is produced by a wide variety of tissues, most notably the adrenal, lung, kidney, and heart. It is also synthesized and secreted in abundance by vascular endothelial cells and is detectable in the circulation. The principal physiological action of AM appears to be as a potent vasodilator, and its systemic administration causes a rapid and profound fall in blood pressure and an increase in pulmonary blood flow. AM has also been found to act as a bronchodilator, an inhibitor of drinking behavior, and an inhibitor of angiotensin-induced aldosterone secretion.

There has been difficulty in defining the receptor sites of AM, and several studies have suggested that its actions are mediated through calcitonin gene-related peptide (CGRP) receptors. There is modest sequence homology between these peptides, in particular the presence of a six residue intramolecular disulfide-linked ring structure that is also present in islet amyloid polypeptide. CGRP has similar vasodilator properties to AM, and this action of AM can be inhibited by the CGRP antagonist, CGRP[8-37]. However, while the specific AM biding sites in rat vascular smooth muscle cells exhibit a K_d of 13 nM, the K_i for CGRP at these sites (300 nM) was significantly greater than that for binding to the CGRP receptor, suggesting that specific receptors for AM do exist and have low affinity for CGRP. Identification of a cDNA clone for such an AM receptor (AM-R) would enhance our understanding of the physiology of this peptide, and facilitate the development of therapeutically useful drugs that could stimulate or block these receptors.

We have recently identified a cDNA clone for the adrenomedullin receptor that was originally isolated as an orphan receptor from rat lung during a search for AT_1 receptor cDNAs. The cDNA encodes a polypeptide of 395 residues that contains seven transmembrane domains and has a general structural resemblance to other members of the G protein-linked receptor superfamily. When expressed in COS-7 cells, this receptor mediates a cAMP response to adrenomedullin with an EC_{50} of 7 nM, and binds ^{125}I-adrenomedullin with a K_d of 8.2 nM, properties that are consistent with those observed in cardiovascular and other AM target tissues. The receptor gene is expressed as several mRNA species, the most prominent of which is a 1.8-kilobase transcript found in the lung, adrenal, heart, spleen, cerebellum, and other sites. Identification of this receptor cDNA should facilitate further investigation of the cellular actions of adrenomedullin and its regulatory effects in normal and disordered states of cardiovascular function.

Ang II-induced signaling and growth responses in glomerulosa cells. Ang II and ACTH are the major hormonal regulators of adrenal function. Ang II controls the growth and differentiation of the zona glomerulose and stimulates aldosterone production, whereas ACTH controls glucocorticoid secretion by the zona fasciculata and also acts in the zona glomerulosa as a potent stimulus of aldosterone secretion. In several Ang II target tissues, AT_1 receptors interact with two distinct guanine nucleotide binding proteins, $G_{q/11}$ and G_i, to activate phospholipase C and to inhibit adenylate cyclase, respectively. We previously demonstrated that in Ang II increased [^{3}H]thymidine incorporation in primary cultures of bovine adrenal glomerulosa cells after first 5 days of culture. At this time, Ang II increased in proportion of cells in S phase and did not cause accumulation of cells in the G_2 phase. Ang II also stimulated the proliferation of glomerulosa cells during treatment for 3 days in the presence of 1% serum, an effect mediated by AT_1 receptors. These results indicate that intracellular mechanisms that mediate growth responses become more active during prolonged culture of glomerulosa cells, and that Ang II exerts mitogenic actions that depend on the functional state of the glomerulosa cells.

Significance to Biomedical Research and the Program of the Institute. Elucidation of the properties of angiotensin receptors and their mechanisms of target-cell activation will contribute to the understanding of normal and disordered mechanisms of body fluid homeostasis and cardiovascular regulation. Ang II receptors are widely distributed throughout the vascular system, adrenal, kidney, and brain, and the clarification of their mechanisms of action at these sites will be facilitated by detailed analysis of the adrenal receptors and their signaling mechanisms. The importance of angiotensin II and its receptors in hypertension and cardiovascular disease has been demonstrated not only in animal models, but also by the therapeutic actions of angiotensin converting enzyme inhibitors and Ang II antagonists in essential hypertension and cardiac failure. Analysis of the structure-function properties of the Ang II receptor and its subtypes should provide new approaches to the design of Ang II antagonists, and to the understanding of mechanisms involved in the actions of Ang II on contractile, secretory, and growth responses in its numerous target tissues.

Proposed Course: The actions of Ang II on early signaling pathways and growth responses, and the structure-function properties of the Ang II receptor, will be further studied in adrenal glomerulosa cells, stably transfected Y_1 cells, and transiently transfected COS cells. The mechanisms by which the Ang II-induced calcium signal, in particular its sustained calcium influx phase, is generated and maintained during agonist stimulation will be investigated in glomerulosa cells and Y_1 cells, with emphasis on the role of Ins(1,4,5)P_3 in the influx process. The manner in which the early calcium signal promotes the expression of c-fos and other early response genes will also be studied in glomerulosa cells. Studies on the growth effects of Ang II in glomerulosa cells will address the activation of mitogenic responses that reflect the trophic action of Ang II on the zona glomerulosa *in vivo*, and the extent to which these are driven by Ang II itself or by other local growth factors in the adrenal gland. The relation between the antagonist and agonist binding sites of the AT_1 receptor will be further investigated by mutational analysis of the external and intra-membrane domains of the receptor. The structural features involved in G protein coupling and internalization will also be evaluated in mutant and native AT_1 receptors. The newly-identified PI 4-kinase that is responsible for maintaining the agonist-sensitive pool of phosphoinositides in several cell types will be purified from the adrenal cortex and its cDNA will be cloned to permit structure-function studies on the enzyme and analysis of factors that regulate its expression.

Publications:

1. Catt KJ. Hormone receptors. In: Hillier SG, Kitchener HC, Neilson JP, eds. Scientific essentials of reproductive medicine. London: WB Saunders, 1996;32-44.

2. Catt KJ. Intracellular signaling. In: Hillier SG, Kitchener HC, Neilson JP, eds. Scientific essentials of reproductive medicine. London: WB Saunders, 1996;45-59.

3. Catt KJ, Hunyady L, Ji H, Tian Y, Baukal AJ, Clark AJL, Balla T. Structure-function properties and signaling pathways of the angiotensin AT_1 receptor. In: Vinson GP, Anderson DC, eds. Adrenal glands, Adrenal glands, vascular system, and hypertension. Bristol: Journal of Endocrinology, 1996; 1-19.

4. Downing, GJ, Kim, S, Nakanishi, S, Catt KJ, Balla, T. Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. Biochemistry 1996;35:3587-3594.

5. Goodfriend TL, Elliott ME, Catt KJ. Angiotensin receptors and their antagonists. New England J Med 1996;334:1649-1654.

6. Hong J, Zheng W, Zheng V, Catt KJ, Sandberg K. Genetic transfer of a nonpeptide binding site to a previously unresponsive angiotensin receptor. Proc Natl Acad Sci USA 1995;92:9240-9244.

7. Hunyady L, Balla T, Catt KJ. The ligand binding site of the angiotensin AT_1 receptor. Trends Pharmacol Sci 1996;17:135-140.

8. Hunyady L, Zhang M, Jagadeesh G, Bor M, Balla T, Catt KJ. Dependence of agonist activation on a conserved apolar residue in the third intracellular loop of the AT_1 angiotensin receptor. Proc Natl Acad Sci USA (in press).

9. Kapas S, Catt KJ, Clark, AJL. Cloning and expression of cDNA encoding a rat adrenomedullin receptor. J Biol Chem 1995;270:25344-25347.

10. Martìnez A, Miller M-J, Catt KJ, Cuttitta F. Adrenomedullin receptor expression in human lung and in pulmonary tumors. (in press).

11. Tian Y, Balla T, Bernstein KE, Sandberg K, Baukal A, Catt KJ. Properties of AT_{1a} and AT_{1b} angiotensin receptors expressed in adrenocortical Y-1 cells. Am J Physiol 1996;270:E831-E839.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00194-08 ERRB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Steroid Biosynthesis and Metabolism in the Mammalian Adrenal Cortex

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C.A. Strott	Head, SSR	ERRB, NICHD
	Y.C. Lee	Staff Scientist	ERRB, NICHD
	H. Akita	Visiting Fellow	ERRB, NICHD
	K. V. Venkatachalam	IRTA Fellow	ERRB, NICHD
	N. Luu	Pre-IRTA Fellow	ERRB, NICHD
	M. Lee	IRTA-Tech	ERRB, NICHD

COOPERATING UNITS *(if any)*

Endocrinology and Reproduction Research Branch

LAB/BRANCH

Section on Steroid Regulation (SSR)

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.0	5.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The biotransformation of steroids by conjugation to a highly charged sulfonate group is an important process that leads to a marked change in the physicochemical properties of these essentially hydrophobic compounds. The sulfonation of steroids is brought about by the transfer of a sulfonate group (SO3-) to an appropriate hydroxyl acceptor site and is carried out by enzymes termed sulfotransferases. Steroid sulfonation, by altering molecular polarity, increases solubility in an aqueous milieu and modifies binding to protein; this in turn influences transportability and acts as an intracellular trapping or storage mechanism. Steroid sulfonation also impacts on biological responsivity by serving to either initiate an event (steroid sulfonate is the active form) or terminate an event (steroid sulfonate is the inactive form). Steroid sulfonation is particularly prominent in steroid-producing tissues; in fact, one of the most active tissues in this regard is the adrenal cortex where tissue-specific steroid sulfotransferases are differentially expressed in functionally distinct adrenocortical zones. The aim of the SSR program is to gain a fuller understanding of the precise biological consequences of steroid sulfonation. To achieve this goal the biochemical characterization and molecular biology of steroid-specific sulfotransferases, as well as ATP-sulfurylase and APS kinase, the catalytic activities responsible for production of the universal sulfonate donor (PAPS), have been undertaken.

The cDNA for stereoselective 3β-hydroxysteroid sulfotransferase (HST) has been cloned, expressed and found to be 87% identical to chiral-specific 3α-HST. The structural genes for estrogen sulfotransferase (EST) and 3β-HST have been determined and the 5'-flanking regions cloned and analyzed; examination of transcriptional regulation is ongoing. An amino acid motif near the C-terminus, conserved in all steroid and phenol sulfotransferases, has been identified as the sulfonate donor binding site; furthermore, amino acid residues involved in estrogen interaction with EST have been specified by site-directed mutagenesis. Human PAPS synthetase, a single polypeptide chain containing both ATP-sulfurylase and APS kinase activities, has been cloned and expressed; additionally, constructs containing either the ATP-sulfurylase or APS kinase domains have been generated and expressed.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

Steroids, primordial molecules that evolved before eukaryotes approximately two billion years ago, constitute one of the earliest forms of information transmitters; they have been described as ancient bioregulators which exerted their message in their cell of origin. Steroids, which make up a subclass of lipids, are part of an extraordinarily large and diverse family of chemical compounds that come under the rubric of terpenes or isoprenoids. This disparate group includes, in part, natural rubber, gutta-percha, numerous fragrant oils, turpentine hydrocarbons, the phytol side chain of chlorophyll, carotenoids and the fat soluble vitamins. What these substances have in common is they are formed by the polymerization of five-carbon isoprene units. Thus, isopentenyl pyrophosphate, the activated isoprene unit, is the building block precursor of the various and sundry terpenes present in nature. In animals isopentenyl pyrophosphate is derived from acetyl CoA and is directed into four important phenyl derivatives: isopentenyl tRNA, ubiquinone, dolichols, and cholesterol along with its steroid derivatives. Steroids, in turn, are subject to various modifications including conjugation reactions. Of the latter, sulfoconjugation is arguably the most important to occur in extrahepatic tissues such as the ovary, testis, adrenal, placenta, intestine and brain.

Sulfoconjugation or sulfonation is a biotransforming mechanism that occurs widely. The sulfonation of drugs and xenobiotics functions primarily to inactivate and clear these generally hydrophobic compounds, although there are examples where the active form of a drug is sulfonated. Importantly, the sulfonation of a host of endogenous compounds is an established metabolic mechanism of immense biological importance. The sulfonation of endogenous substances covers an extended range of molecules of diverse size, structure, chemistry and function. Membrane and secretory proteins are strategically modified by post-translational sulfonation. Macromolecules such as glycosaminoglycans and proteoglycans, components of cell surface and connective tissue structures, are subjected to essential modifications by the addition of sulfonate groups. Importantly, a multitude of low molecular weight compounds including neurotransmitters, catecholamines, iodothyronines and steroids are modified by sulfoconjugation.

The sulfoconjugation of steroids is carried out by sulfotransferases, cytosolic enzymes that transfer a sulfonate radical (SO_3^-) from an activated sulfonate donor molecule to an appropriate hydroxyl acceptor site. Sulfonation brings about a striking change in the physicochemical property of steroids as a result of insertion of the highly charged sulfonate moiety. With pKas < 1.5, steroid sulfonates remain fully ionized at all biological pH levels. By altering molecular polarity, steroid sulfonation increases solubility in aqueous milieus and modifies binding to protein; this in turn influences transport and acts as an intracellular trapping or storage mechanism. Sulfonation also impacts on the biological responsivity of steroids by serving to either initiate or terminate actions when serving as the active or inactive form, respectively. Additionally, steroid sulfonates can serve as substrates for P_{450} steroidogenic enzymes. These material considerations, notwithstanding, a complete understanding of steroid sulfonation, especially in extrahepatic tissues remains to be realized.

An important aspect of the sulfonation process is concerned with the availability of the sulfonate donor molecule. In mammals, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the universal activated sulfonate donor, is synthesized from ATP and inorganic sulfate by the concerted action of ATP sulfurylase and adenosine 5'-phosphosulfate (APS) kinase (PAPS synthetase). The availability of PAPS, as well as the activity of individual sulfotransferases, limits the rate of sulfonation, particularly when the sulfate ion concentration is in the physiologic range. Notwithstanding the fact that sulfonation is a ubiquitous process, little information is available on the production and metabolism of PAPS in any tissue.

In an effort to gain a fuller understanding of the intriguing steroid sulfonation biotransforming process, emphasis is currently placed on the isolation, characterization and cloning of individual members of the steroid sulfotransferase family along with PAPS synthetase. The cloning of cDNAs and generation of specific antibodies will initiate numerous experiments dealing with gene structure, transcriptional regulation, mRNA metabolism, cell-specific expression and development. Eventually, with the use of knockout and/or antisense technologies, the biological import of these poorly understood enzyme systems can be more definitively determined.

Major Findings:

Steroid sulfotransferase active site. Steroid sulfotransferases interact with two substrates, the sulfonate donor and the steroid acceptor molecules. A GxxGxxK sequence, related to the P-loop motif commonly found in ATP- and GTP-binding proteins, is positioned within a highly conserved region located near the C-termini of all steroid and phenol sulfotransferases for which primary structures are known. To examine the GxxGxxK motif in detail for its involvement in the binding of PAPS, point mutations that replaced the conserved glycines (G260 and G263) and lysine (K266) with alanines were introduced singly or in combination into guinea pig estrogen sulfotransferase (gpEST) cDNA. Additionally, as "controls," non-motif residues S262, D264 and N267 were also mutated to alanine, singly or in combination. Nine mutant constructs generated from wild type gpEST cDNA were all found to express the 34 kDa gpEST protein as verified by immunoblot analysis. The expressed proteins were subjected to kinetic analysis to detect perturbations in their interaction with both PAPS and the estrogen substrate. While the mutation of a single motif residue had little effect on the interaction between gpEST and PAPS as determined by kinetic analyses as well as photoaffinity labeling, the mutation of any two residues in concert resulted in an approximate 10-fold increase in the K_m for PAPS and reduced photoaffinity labeling. The mutation of all three motif residues resulted in an inactive enzyme and complete loss of photoaffinity labeling, although the protein was expressed in a reasonable amount. On the other hand, the K_m for PAPS was unchanged from wild type with either the double or triple "control" mutants, demonstrating the singular importance of the motif residues specifically. Several mutant constructs also displayed a striking effect on the K_m for the estrogen substrate with double motif mutants, again, demonstrating greater perturbations (8- to 28-fold increase in K_m) than single mutations. Whereas the mutation of non-motif residues had a negligible effect on the K_m for PAPS, there was a marked increase in the K_m for the estrogen substrate (>30-fold). The data indicate that G260, G263, and K266, amino acid residues, that constitute the GxxGxxK P-loop motif, play a critical role in the interaction between estrogen sulfotransferase and the universal sulfonate donor, PAPS. Additionally and importantly, the specific residues G260 and N267 are crucially involved in the ability of the enzyme to efficiently sulfonate the estrogen substrate. These novel findings indicate that the sequence GISGDWKN, encoded by the 5'-end of the 3' terminal exon of the gpEST gene, represents an essential component of the active site for gpEST and that, in so much as the GxxGxxK motif is universally conserved, this area will also prove to be catalytically important for all steroid and phenol sulfotransferases.

Molecular cloning of a second 3-hydroxysteroid sulfotransferase. A second guinea pig adrenal 3-hydroxysteroid sulfotransferase (gpHST2) cDNA has been cloned that is distinct from the previously cloned chiral-specific 3α-hydroxysteroid sulfotransferase (gp3αHST now designated gpHST1). The deduced amino acid sequences for gpHST1 and gpHST2 are 86% identical; however, whereas gpHST1 stereoselectively acts on 3α-hydroxylated neutral steroids gpHST2 demonstrates a clear preference (but not exclusive specificity) for 3β-hydroxylated steroids. Thus gpHST2 is similar to a previously isolated gpHST that selectively acted on 3β-hydroxylated steroids (gp3βHST). Additionally gpHST2 at 33 kDa is the same size as gp3βHST and larger than gpHST1 (32 kDa). gpHST2 also contains amino acid sequences identical to peptides obtained from gp3βHST and cross-reacts with antibodies raised against purified gp3βHST. Nonetheless, gpHST2 can sulfonate both 3α-and 3β-hydroxylated neutral steroids suggesting that either gp3βHST does not have the exquisite stereoselectivity previously indicated or this subfamily of hydroxysteroid sulfotransferases is larger than originally thought.

Isolation and characterization of guinea pig PAPS synthetase. The activation of inorganic sulfate to form PAPS results from the concerted actions of ATP sulfurylase (SUL) (reaction #1) and APS kinase (KIN) (#3):

1) $\text{ATP} + \text{SO42-} \rightarrow \text{APS} + \text{PP}_i$
2) $\text{PP}_i + \text{APS} \rightarrow \text{ATP} + \text{SO}_4\text{2-}$
3) $\text{APS} + \text{ATP} \rightarrow \text{PAPS} + \text{ADP}$

Reaction #1 is thermodynamically unfavorable in the forward direction; therefore, to assay for this enzyme, the more favorable reverse reaction is used (reaction #2). Thus, $[^{35}S]$APS and radioinert pyrophosphate (PP_i) are used to measure the reaction by quantifying the formation of $[35SO_4]$. APS kinase activity is measured by the formation of radioactive PAPS using $[^{35}S]$APS and ATP (reaction #3). In isolating SUL and KIN from guinea pig adrenal cytosol it was found that the two activities co-purified throughout the purification scheme which involved ammonium sulfate precipitation (55-65%), S-200 gel filtration, ion-exchange chromatography and PAP-affinity chromatography. The fraction containing the coincident peak activities eluted from the affinity gel revealed a single protein band of 55 kDa when analyzed by SDS gel electrophoresis and silver staining. Isoelectric focusing of the same preparation used for SDS gel analysis revealed two bands with pIs of 6.8 and 6.9 suggesting charge isoforms. The bifunctional activity of the purified 55 kDa protein (PAPS synthetase) is in contrast to the situation in bacteria, fungi, yeast and plants where SUL and KIN are associated with distinct proteins. Interestingly, it was noted that during purification inorganic sulfate was consistently formed, even with the most highly purified preparation. While this raised the possibility of an intrinsic PAPS sulfatase activity, it was initially presumed to result from either contamination with a sulfatase or possibly the backward reaction was occurring (reaction #2) as a result of PPi contaminating the ATP preparation. In regards to the first possibility, aside from obtaining further proof of protein purity, it was reasoned that if indeed there is a contaminating sulfatase it would utilize exogenous PAPS and form inorganic sulfate in the presence of the same cofactors, metal ions and assay conditions. Thus, when exogenous PAPS was used as substrate, only a minuscule amount of PAPS hydrolysis could be detected suggesting that a contaminating sulfatase was not present in the purified preparation. In regards to the second possibility, treatment of the purest preparation of ATP with pyrophosphatase (to degrade any PP_i that might possibly be present) prior to enzymatic analysis did not alter the formation free sulfate. It was concluded that the inability of the most purified preparation to utilize exogenous PAPS to form sulfate and the failure of pyrophosphatase to alter the results suggested that PAPS sulfatase activity might indeed be intrinsic to PAPS synthetase. When KIN was assayed in the presence of 250 µM ATP, the [^{35}S]APS substrate was quantitatively converted to [^{35}S]PAPS with no free [$^{35}SO_4$] being formed. Interestingly, however, when 5 mM ATP was used in the assay, PAPS formation was essentially undetectable and [$^{35}SO_4$] accumulation occurred instead. Thus, the intrinsic PAPS sulfatase activity of the most pure preparation of PAPS synthetase was dependent on the concentration of ATP. When[^{3}H]APS was used as the radioactive substrate and the reaction products analyzed by PEI-TLC, no [^{3}H]AMP was formed ,eliminating ATP-dependent APS sulfatase activity. Thus, the sulfatase activity intrinsic to purified PAPS synthetase is a PAPS-specific sulfatase that is ATP-dependent. At this juncture PAPS synthetase appears to be a multifunctional protein responsible for the production of two compounds of importance in sulfotransferase reactions, namely PAPS and PAP (3',5'ADP): the former being essential for sulfonation as the universal sulfonate donor molecule; the latter has two apparent roles. We have previously reported that PAP is a potent inhibitor of sulfonation by EST, a reaction that involves a basic isoform of EST; at the same time PAP promotes high affinity estradiol binding to an acidic isoform of EST. This level of biochemical and physiological complexity is not easily understood in our current state of knowledge regarding these intriguing enzyme systems.

Cloning and expression of human PAPS synthetase. Expressed sequence tags (est) from a directionally cloned human infant brain cDNA library were searched for a clone with sequence homology to yeast APS kinase. Such a clone (est07073) was obtained from ATCC (HIBBQ-19). Since a 480 bp stretch of the 5'-end of HIBBQ-19 had been sequenced, the deduced 160 amino acid sequence was available. The HIBBQ-19 clone was amplified and the plasmid isolated for use as a sequencing template. A sense primer based on the conserved FISP region of APS kinase in E. coli and S. cervisiae was synthesized and used for sequencing. By contiguous cDNA walking using eight overlapping sequence primers, a continuous sequence of 2098 bp was obtained to the poly-A tail. The 5'-end was completed using the 5'-RACE procedure. The full-length human PAPS synthetase (hPAPSS) cDNA consists of 2221 bp. Cytosol prepared from COS-1 cells transfected with the hPAPSS cDNA contained significant activities (2-3 x) for ATP sulfurylase and APS kinase over background compared to nontransfected cells demonstrating bifunctionality for the expressed protein.

ATP sulfurylase and APS kinase have been cloned from bacteria, fungi, yeast, and plants, where the enzymes are found on separate polypeptide chains. In these species the Mr of ATP sulfurylase ranges from 35.2 to 63.9 kDa, while the Mr of APS kinase ranges from 22.3 to 29.7 kDa. Interestingly, in contrast to what is found in bacteria, fungi, yeast and plants, ATP sulfurylase and APS kinase isolated from mammalian tissues, such as rat chondrosarcoma tissue and the guinea pig adrenal (vide supra), are physically linked on a single bifunctional protein (Mr 56 kDa and 55 kDa, respectively). In confirmation of this important finding, a cDNA was recently cloned from a marine worm that expresses a 57 kDa protein containing both ATP sulfurylase and APS kinase activities. Subsequently, a mouse cDNA was cloned expressing a correspondingly bifunctional PAPS synthetase protein.

Worm PAPS synthetase has a calculated molecular mass of 68397 daltons and pI of 6.23; mouse PAPS synthetase has a calculated molecular mass of 70713 daltons and a pI of 6.30; and human PAPS synthetase has a calculated molecular mass of 61621 daltons and a pI of 6.40. Amino acid sequence alignment of the three proteins reveals 362 identities (58%). The worm and human PAPS synthetases are 59% identical (identities increase to 68% when length differences are considered). Remarkably, the mouse and human PAPS synthetases are 86% identical (again, because of length differences, identities rise significantly to 96% when this is factored in). Clearly, a marked conservation of structure exists for PAPS synthetase as illustrated here for such widely spaced species as worms, mice and human beings. An interesting structural feature of the three PAPS synthetase proteins is that each contains a nucleotide-binding motif near their N-termini termed a P-loop described for ATP- and GTP-binding proteins. The primary structure of a P-loop typically consists of a glycine-rich region followed by a conserved lysine. Interestingly, an identical P-loop motif is conserved for APS kinase in bacteria, yeast and plants; on the other hand, ATP sulfurylase from bacteria, yeast, fungi and plants does not contain a similarly conserved motif. It is of further interest that a related P-loop motif is present in all steroid and phenol sulfotransferases cloned to date (vide supra).

The fusion of ATP sulfurylase and APS kinase into a single polypeptide chain would seem to provide an adaptive advantage by facilitating the "channeling" of APS from ATP sulfurylase to APS kinase. This rationale is essentially based on the following considerations: the substantial energy barrier to APS formation (vide supra), and the purported instability of APS in cells. Thus, development of a *fused* protein system represents an evolutionary step creating a more efficient pathway that is able to overcome inherent obstacles to the synthesis of activated sulfate and production of this crucial universal sulfonate donor molecule.

Significance to Biomedical Research and the Program of the Institute:

It is well established that steroid hormones secreted by the mammalian adrenal cortex play a crucial role in stress and immune responses. In addition, adrenocortical steroids can markedly influence reproduction and have a profound affect on normal growth and development. All of the aforementioned processes are subject to intense biomedical research and of great importance to the program of the Institute.

The adrenal cortex is the sole source of two potent steroid hormones, cortisol and aldosterone, hormones essential for maintenance of the *internal milieu* and a normal healthy state. Cortisol, the principal glucocorticoid, is known to be secreted in increased amounts in response to stressful stimuli; however, its 'basal' biological function is not well understood. Aldosterone is the principal mineralocorticoid or salt-retaining steroid hormone. It is thus important to understand how the adrenal cortex responds to various effectors, and how the biosynthesis and metabolism of adrenocortical steroids are influenced and modulated. In actuality, the adrenal cortex produces a large array of steroid compounds, some of which are structurally and functionally related to either glucocorticoids or mineralocorticoids, while others are structurally and functionally related to the sex steroids estrogens and androgens. The biological significance of the local production of bioactive steroids in the adrenal cortex is not presently appreciated. Additionally, the adrenal cortex actively modifies steroids by the process of sulfonation and the biological importance of this fact is also not understood. The adrenal cortex is, in fact, a complex endocrine organ system and the level of complexity is increased by the realization that it does not consist of a homogeneous population of cells, but rather multiple populations that are more or less arranged into concentric regions or zones. While there is a substantial amount of information regarding the outermost zona glomerulosa (source of aldosterone) and the middle zona fasciculata (source of cortisol), it is rather astonishing that, to date, the functional status of the innermost zona reticularis remains an enigma. In the human being, the zona reticularis is rudimentary and essentially undeveloped until puberty, at which time it begins to form. Associated with this evolvement is a dramatic increase in the production and secretion of dehydroepiandrosterone and its sulfonated form, a steroid structurally classified as an androgen. The regulatory elements involved in this phenomenon have yet to be determined.

Do steroid sulfonates have an intrinsic biological function? In the nongenomic action of steroids, in contrast to the genomic theory, a classical steroid receptor is not required; that is, steroids act directly at extranuclear sites, usually on membranes. In the nongenomic version of steroid action there are several examples where steroid sulfonates themselves rather than their unconjugated counterparts, exhibit a direct action. Sulfoconjugates of estrogens in very low concentrations competitively interfere with the activation of kynurenine transaminase by its coenzyme, pyridoxal 5-phosphate, and it has been postulated this represents a general interaction of conjugated steroids in regulating coenzyme-apoenzyme association. Estradiol sulfonate, but not unconjugated estradiol, is a potent inhibitor of glutathione S-transferase, a key enzyme in protection against metabolic stress. Estrone sulfonate directly produces ultrastructural changes and has a specific effect on protein synthesis and secretion by endometrial glandular cells in primary culture. The activity of phospholipase A_2 in amniotic membranes is enhanced by the sulfonates of dehydroepiandrosterone and pregnenolone but not their unconjugated forms. Sterol and steroid sulfonates, by stabilizing the outer membrane of spermatozoa, have an inhibitory effect on acrosin activity and thus capacitation and fertilization. In the central nervous system, pregnenolone sulfate and dehydroepiandrosterone sulfate have been shown to be potent antagonists of benzodiazepine-GABA$_A$ receptors that regulate chloride channels and thus are neuroexcitatory. In this regard, it is of interest that benzodiazepine receptors are also present in peripheral tissues, a particularly rich source being the adrenal cortex where they have been found to be located on outer mitochondrial membranes. This finding is potentially important because mitochondria are the site of the rate-limiting step in steroidogenesis, i.e. the conversion of cholesterol to pregnenolone. Sulfoconjugates of estrogenic steroids have emerged as an important form of these compounds. In the human being, the most abundant circulating estrogen is estrone sulfate, which in both men and women is present in a 10- to 15-fold greater concentration than the unconjugated form of either estrone or estradiol. In target tissues, estrone sulfate can be converted to unconjugated estrone by an estrone sulfate-specific sulfatase and the free estrone subsequently reduced to biologically active estradiol. Breast cancer cells derive a significant amount of unconjugated estrogen from extracellular estrone sulfate. Furthermore, in post-menopausal women, plasma estrone sulfate serves as the major source of estradiol available to breast cancer cells. Importantly, the sulfoconjugation of estrogens in the uterus during the menstrual cycle as well as in normal breast tissue is under the control of progesterone and/or estradiol and serves to regulate the level of biologically active free hormone. Finally, we have reported that a high concentration of EST immunoreactivity is present in nuclei of the guinea pig adrenal cortex, suggesting that EST might play a role in modulating the ability of active estrogens to regulate gene expression in ACTH-responsive cells.

Proposed Course:

A. Completion of the exon/intron composition for chiral-specific 3β-HST. A 2.5 kb segment of the 5'-flanking region of the 3β-HST gene has been cloned; and while the 3β-HST structural gene is almost complete, splice sites for intron IV remain to be established.

B. Examination of the 3β-HST gene promoter activity by generating constructs of the 5'-flanking region containing block deletions fused to a reporter gene. This will be followed by gel-shift analyses and DNA foot-printing.

C. Expression and kinetic analyses of separate constructs of human PAPS synthetase consisting of the APS kinase domain (N-terminus) and the ATP sulfurylase domain (C-terminus).

D. Site-directed mutagenesis of human PAPS synthetase to determine structural correlates involved in the binding of ATP and catalytic activity.

E. Cloning and expression of the guinea pig PAPS synthetase cDNA.

F. Overexpression of EST to obtain material for biochemical characterization and crystallization.

G. Studies of EST transcriptional regulation.

H. Site-directed mutagenesis to examine structural determinants for 3α/3β-HST enantioselectivity.

I. Screening of guinea pig adrenal cDNA library to isolate testosterone sulfotransferase.

Publications:

Chiba H, Komatsu K, Lee YC, Tomizuka T, Strott CA. The 3'-terminal exon of the family of steroid and phenol sulfotransferase genes is spliced at the N-terminal glycine of the universally conserved GxxGxxK motif that forms the sulfonate donor binding site. Proc Natl Acad Sci USA 1995;92:8176-8179.

Driscoll WJ, Komatsu K, Strott CA. Proposed active site domain in estrogen sulfotransferase as determined by mutational analysis. Proc Natl Acad Sci USA 1995;92:12328-1233.

Luu NX, Driscoll WJ, Strott CA. Molecular cloning and expression of a guinea pig 3-hydroxysteroid sulfotransferase distinct from chiral-specific 3α-hydroxysteroid sulfotransferase. Biochem Biophys Res Commun 1995;217:1078-1086.

O'Malley BW, Strott CA. Steroid hormones: metabolism and mechanism of action. In: Yen SSC, Jaffe RF, Barbieri RL, eds. Reproductive Endocrinology, 4th Ed. WB Saunders (in press).

Strott CA. Steroid sulfotransferases. Endocr Rev (in press).

Venkatachalam KV, Strott CA. Human PAPS synthetase: a bifunctional protein that catalyses formation of the universal sulfonate donor molecule. Gene (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD00195-03 ERRB

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Intracellular Signaling in Endocrine Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S.S. Stojilkovic	Head, UCS	ERRB, NICHD
Others:	G. Bonhomme	Pre-IRTA Fellow	ERRB, NICHD
	T. Koshimizu	Visiting Fellow	ERRB, NICHD
	A. Lachowich	Visiting Fellow	ERRB, NICHD
	M. Tomic	Visiting Associate	ERRB, NICHD
	L. Zheng	NRC Fellow	ERRB, NICHD

COOPERATING UNITS *(if any)*
Laboratory of Cell Biology and Genetics, NIDDKD (E. Rojas)
Mathematical Research Branch, NIDDK (J. Rinzel)
Institute of Theoretical Dynamics, University of California, Davis (J. Keizer)

LAB/BRANCH
Unit on Cellular Signaling

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The integration of voltage-sensitive and -insensitive calcium entry pathways in gonadotropin-releasing hormone (GnRH)-induced calcium mobilization and luteinizing hormone (LH) release were investigated in anterior pituitary gonadotrophs. Increase in calcium entry through voltage-sensitive calcium channels in nystatin-perforated cells is associated with two distinct positive effects on GnRH-evoked calcium release. It facilitates a decrease in the interspike periods that lead to increase in the frequency of spiking and also magnifies calcium signaling by increasing the duration and amplitude of calcium oscillations. The same effects are observed in cells in which calcium oscillations are induced by $InsP_3$. Pituitary gonadotrophs also express ATP-gated receptor channels. Their activation causes calcium influx and a consequent increase in cytosolic calcium concentrations. Purinergic channels are voltage-insensitive, but their activation is associated with the depolarization of gonadotrophs and the additional facilitation of calcium entry through voltage-sensitive calcium channels. Purinergic channel-mediated calcium influx also affects agonist-induced and $InsP_3$-dependent calcium oscillations by increasing the frequency, baseline, and duration of spiking. The effects of voltage-sensitive and ATP-gated calcium channels on the frequency of calcium spiking are consistent with the model simulation of calcium spiking, in which a small increase in cytosolic calcium concentrations is able to trigger a large pulse of calcium oscillations in the presence of a constant $InsP_3$ concentration. Both calcium entry pathways also potentiate GnRH-induced LH release. Gonadotrophs were found to co-secrete ATP with LH, indicating the physiological significance of the expression of purinergic channels in these cells. In perifused pituitary cells, ATP is promptly degraded by ecto-ATPase, ecto-ADPase, and ecto-5' nucleotidase to adenosine in a calcium/magnesium-dependent manner. These observations indicate that ATP represents a positive feedback element in agonist-induced calcium signaling and gonadotropin secretion, and that this action is controlled by ectonucleotidases.

Project Description

Objectives:

To clarify the cellular signaling cascade in endocrine and neuroendocrine cells operated by calcium-mobilizing receptors, and the interactions between plasma membrane electrical events and receptor-mediated signaling. In particular, to characterize the spatial and temporal patterns of phospholipase C- and phospholipase D-dependent intracellular signaling, and the interactions between signaling molecules triggered by these two pathways in the control of secretion and gene expression. The research includes studies on the electrophysiological characterization of spontaneous and agonist-induced plasma membrane and endoplasmic reticulum (ER) membrane excitabilities, the patterns and mechanisms of calcium oscillations, the dependence of inositol 1,4,5-trisphosphate ($InsP_3$)-controlled cytosolic calcium oscillations on membrane potential (V_m), and the coordinate actions of phospholipase C- and phospholipase D pathways in the control of hormone secretion and the expression of early response genes.

Methods Employed:

Dispersion and culture of pituitary cells. αT3-1, GT1-7, and GH_3 cell line cultures. Photometry and imaging techniques for measurements of cytosolic calcium concentrations ($[Ca^{2+}]_i$) in single cells. Whole cell patch clamp studies in pituitary gonadotrophs. Inositol phosphate, diacylglycerol, cytidine diphosphate diacylglycerol, phosphatydylethanol, and phosphatidic acid measurements. RNA and Northern blot analysis of c-fos, c-jun, and junB. Radiolabeled ligand binding studies. Mathematical modeling of the data.

Major Findings:

1. Expression of Purinergic Receptor Channels in Pituitary Cells. Adenosine trisphosphate (ATP) is frequently stored in secretory granules together with other neurotransmitters and hormones, and is often co-secreted during agonist stimulation. Once released into the extracellular medium, ATP may bind to its plasma membrane receptors (P_2 purinergic receptors), leading to increases in $[Ca^{2+}]_i$, and potentiation of exocytosis. ATP receptors belong to two major groups: G protein-coupled receptors (P_{2Y}), whose activation causes Ca^{2+} release from its intracellular stores, and plasma membrane receptor-channels (P_{2X}), whose activation promotes Ca^{2+} influx. Thus, the action of ATP during exocytosis may represent a positive feedback mechanism or self-potentiation of the calcium signaling and secretion (autocrine regulation). In addition, ATP may diffuse and activate P_2 receptors in neighboring cells (paracrine regulation). On the other hand, ATP cannot act as a typical hormone since it is rapidly degraded by several ecto-ATPases.

Calcium-mobilizing P_{2Y} receptors have previously been identified in pituitary cells. We have recently observed that rat pituitary gonadotrophs express P_{2X} receptor-channels. These channels were activated by ATP and ADP, but not AMP or adenosine. ATP induced a dose- and extracellular Ca^{2+}-dependent rise in $[Ca^{2+}]_i$ in identified gonadotrophs in Mg^{2+}- and suramin-sensitive manner. ATP, adenosine-5'-0-(3-thiotriphosphate), adenosine-5'-0-(1-thiotriphosphate), 2-methylthio-ATP, and 3'-0-(4 benzoyl)benzoyl-ATP were roughly equipotent in rising $[Ca^{2+}]_i$ in gonadotrophs, while ADP was effective only at submilimolar concentration range, and none of these compounds permeabilized the cells. On the other hand, α,β-methylene-ATP, β,γ-methylene-ATP, and UTP were unable to induce any rise in $[Ca^{2+}]_i$. This pharmacological profile is consistent with expression of $P2X_2$ and/or $P2X_5$ purinergic receptor-channels in gonadotrophs. In addition, ATP stimulated gonadotropin secretion and enhanced agonist-induced gonadotropin release. These observations suggest that ATP represents a paracrine/autocrine factor in the regulation of Ca^{2+} signaling and secretion in gonadotrophs, and that its actions are mediated by P_2 receptor-channels.

2. Secretion and Degradation of ATP in Pituitary Cells. To further clarify the role of ATP as a positive feedback element in agonist-induced calcium signaling and gonadotropin release, an analysis of ATP secretion and degradation was performed in a mixed population of pituitary cells. However, under conditions normally used to measure LH release, no measurable ATP was secreted from gonadotropin-releasing hormone (GnRH)-

stimulated cells. Further experiments revealed that the detection of ATP released from GnRH-stimulated cells was prevented by its rapid degradation in the presence of pituitary cells. To permit the measurement of ATP secretion, several ecto-ATPase inhibitors were employed, including N-ethyl maleimide, trifluoroperazine, and ethacrinic acid. Also, ATP secretion was examined in cells perifused with Ca^{2+}/Mg^{2+}-deficient medium, since it has been reported that these ions are required for activation of ecto-ATPases. Only a minor reduction of ecto-ATPase activity was observed in cells treated with these inhibitors or in cells perifused with Ca^{2+}/Mg^{2+}-deficient medium. However, the presence of Ca^{2+}/Mg^{2+} chelators, EGTA or EDTA, in the medium significantly inhibited ATP degradation, and an obvious GnRH-induced ATP co-secretion with LH was observed, with a 10 : 1 ratio. In both calcium-containing and -deficient conditions, only a partial recovery of ATP was observed after employing myokinase/pyruvate kinase enzymatic reactions, which convert AMP and ADP to ATP. In contrast, the recovery of ATP by myokinase/pyruvate kinase reactions was almost complete in perifused pituitary cells exposed to α,β-MeS-ADP, a specific inhibitor of 5'- nucleotidase. These observations are consistent with the conclusion that pituitary cells express functional ecto-ATPase, ADPase, and 5'-nucleotidase, which rapidly degrade ATP in a sequential manner to adenosine, leading to the termination of ATP action as an amplifier of agonist-induced Ca^{2+} signaling and secretion.

3. Ca^{2+} Excitability of the Endoplasmic Reticulum Membrane. In many cell types expressing Ca^{2+}-mobilizing receptors, oscillations and waves in $[Ca^{2+}]_i$ occur as a part of the signal transduction process for extracellular agonists. In several well characterized systems, such as *Xenopus laevis* oocytes, hepatocytes, and pancreatic acinar cells, the generation of oscillations in the $[Ca^{2+}]_i$ is explained as a consequence of calcium release through $InsP_3$ receptor (IP_3R) channels. Current hypotheses favor the view that $[Ca^{2+}]_i$ oscillations arise as an outcome of the different kinetics of activation and inactivation intrinsic to the IP_3R channel, working in conjunction with the ER Ca^{2+}-ATPase. The critical points in these models are that a fluctuating Ca^{2+} signal originates in response to the continuous occupancy of plasma membrane receptors and a non-oscillatory production of $InsP_3$, and that calcium itself is responsible for the generation of oscillations. In accord with the first requirement, we have previously shown that agonist-induced oscillations in $[Ca^{2+}]_i$ can be mimicked by injection of non-metabolizable $InsP_3$ analogs. During the last year, we have theoretically and experimentally addressed the stimulatory role of calcium on its own release from the ER, using pituitary gonadotrophs as a cell model. In collaboration with Drs. Yue-Xian Li and John Rinzel, Mathematical Research Branch, NIDDK, and Dr. Joel Keizer, Institute of Theoretic Dynamics and Department of Chemistry, University of California at Davis, a previously developed mathematical model that describes $[Ca^{2+}]_i$ oscillations in pituitary gonadotrophs was further advanced to emphasize the role of calcium in the initiation and regeneration of calcium transients during continuous $InsP_3$ stimulation. In this model, a small increase in $[Ca^{2+}]_i$ is able to trigger a large pulse of $[Ca^{2+}]_i$ oscillations, and such a feature of the ER is termed ER calcium excitability. This dynamic property of the ER membrane manifests itself only when $InsP_3$ is above its basal level, and is linked to the gating properties of IP_3R channels combined with ER calcium pump activity.

4. Modulation of the Kinetics of $InsP_3$-Induced $[Ca^{2+}]_i$ Oscillations by Calcium Entry. To study the role of Ca^{2+} in $InsP_3$-induced Ca^{2+} release experimentally, we explored the influence of voltage-sensitive Ca^{2+} entry on kinetics of $[Ca^{2+}]_i$ oscillations triggered by GnRH in cultured rat gonadotrophs. In collaboration with Drs. Leoncio Vergara and Manuel Kukuljan, University of Chile, we have evaluated the influence of sustained changes in the rate of Ca^{2+} entry on the pattern of agonist/ $InsP_3$-induced Ca^{2+} spiking. Nystatin-perforated and whole-cell modalities of the patch-clamp technique were employed. The use of the nystatin-perforated patch on excitable gonadotrophs makes it possible to rapidly and transiently modulate $[Ca^{2+}]_i$ by manipulating V_m under voltage-clamp conditions. Since the nystatin-formed pores are not permeable to calcium, $[Ca^{2+}]_i$ is not influenced by the content of this cation or by the Ca^{2+} buffering compounds in the pipette solution. In addition, the pattern of Ca^{2+} spiking can be analyzed in the absence of the buffering effects of fluorescent dyes by employing the apamin-sensitive Ca^{2+}-operated potassium current ($I_{K(Ca)}$) as a $[Ca^{2+}]_i$ indicator. Furthermore, in the whole-cell patch-clamp configuration the cytosolic $InsP_3$ concentration can be held constant by continuous diffusion from the pipette solution. Finally, this system also provides the possibility for simultaneous measurements of V_m & $[Ca^{2+}]_i$ oscillations or current & $[Ca^{2+}]_i$ oscillations. Thus, although large cells such as oocytes provide greater flexibility for measuring and manipulating the $InsP_3/Ca^{2+}$-controlled Ca^{2+} release, the expression of voltage-sensitive calcium channels (VSCC) in the plasma membrane of small cells such as

gonadotrophs makes them also suitable for studying the role of cytosolic Ca^{2+} in agonist/$InsP_3$-induced Ca^{2+} spiking.

The elements of the pattern of Ca^{2+} oscillations employed in the characterization of ER Ca^{2+} excitability were the frequency and amplitude of Ca^{2+} spiking, and the duration of the spikes (*time-on*) and the interspike periods (*time-off*). In physiological conditions, the frequency of the GnRH-induced oscillations increased with time, while the amplitude decreased until both reached stable values. However, in cells with V_m held at -50 mV or below, both parameters progressively decreased until the signal was abolished. These effects were reverted by a depolarization of the membrane positive to -45 mV in both agonist- and $InsP_3$-stimulated gonadotrophs. Depolarization also led to an increase in the fraction of time during which $[Ca^{2+}]_i$ remained elevated. This effect originated from both an increase in *time-on* and a decrease in the *time-off*. The frequency and amplitude of spiking as well as the *on* and *off times* depended on the activity of VSCC, but displayed different temporal courses and voltage relationships. The depolarization-driven recovery of the frequency was instantaneous, while the recovery of amplitude of spiking was more gradual. The midpoints of the V_m sensitivity curve for *time-on* and amplitude of spiking (-15 mV) were close to the values observed in the L-type Ca^{2+} current and in a depolarization-induced increase in $[Ca^{2+}]_i$, while this parameter was much lower (-35 mV) for *time-off* and frequency of spiking. These observations indicate that Ca^{2+} entry exhibits two distinct positive feedback effects on $InsP_3$-evoked Ca^{2+} release. It facilitates Ca^{2+} liberation due to its direct and instantaneous action on the release mechanism, as manifested by a decrease in *time-off* and a consequent increase in the frequency of spiking. It also magnifies the Ca^{2+} signal due to its gradual effect on the balance between the efflux and influx of Ca^{2+}, as indicated by an increase in *time-on* and amplitude of Ca^{2+} oscillations.

5. Integration of P_2 Channels in Plasma Membrane- and ER-Derived Calcium Oscillations. The observation that pituitary cells express an additional ATP-gated calcium channel was also employed in the characterization of the stimulatory role of calcium on its own release from the ER. Patch-clamp experiments showed that ATP induced an inward depolarizing current in cells clamped at -90 mV, which is associated with an increase in $[Ca^{2+}]_i$. The ATP-induced $[Ca^{2+}]_i$ response was partially inhibited by nifedipine, a blocker of VSCC, but was not affected by tetrodotoxin, a blocker of voltage-sensitive Na^+ channels. Thus, the P_2-depolarizing current itself not only drives Ca^{2+} into the cell, but also activates Ca^{2+} entry through VSCC. In accord with this, low [ATP] induced plasma membrane-dependent $[Ca^{2+}]_i$ oscillations in quiescent cells, and increased the frequency of spiking in spontaneously active cells. ATP-induced Ca^{2+} influx also affected agonist-induced and $InsP_3$-dependent $[Ca^{2+}]_i$ oscillations by increasing the frequency, baseline, and duration of Ca^{2+} spiking. These observations indicate that ATP modulates both the plasma membrane and ER oscillator. The effects of ATP on agonist-induced calcium spiking are consistent with the bidirectional effects of calcium on $InsP_3$-controlled calcium release.

Significance to Biomedical Research and the Program of the Institute:

The clarification of receptor and channel-mediated intracellular signaling is essential for the understanding of both normal and disordered cellular functions. Current emphasis is on the characterization of agonist- and plasma membrane calcium channel-mediated Ca^{2+} signaling and Ca^{2+}-controlled cellular functions. In general, Ca^{2+} is involved in the activation of a complex set of intracellular enzymes, gene expression, hormone and neurotransmitter release, and cell division and differentiation. Furthermore, cytosolic Ca^{2+} participates in intracellular "crosstalk", i.e., is involved in activation of the other intracellular signaling systems such as the phospholipase D and phospholipase A_2 signaling pathways. The complex pattern of interactions between Ca^{2+} and protein kinase C signaling in controlling cellular functions, including the expression of early response genes and hormone secretion, further indicates the need for a comparative analysis of intracellular signaling in agonist-stimulated cells. Thus, the regulation of calcium mobilization from an intracellular store(s) and calcium entry through a variety of plasma-membrane calcium channels are of central importance in the cellular physiology of endocrine and neuroendocrine cells.

Proposed Course:

1. Sensing and Refilling Calcium Stores in Pituitary Gonadotrophs. During agonist-induced Ca^{2+} mobilization the content of intracellular Ca^{2+} stores declines, resulting in increased Ca^{2+} entry via a process known as capacitative calcium entry. In a number of nonexcitable cells, capacitative entry is believed to occur through a Ca^{2+}-release-activated Ca^{2+} channel. Several hypotheses have been proposed to explain how the store content regulates these channels, ranging from the existence of diffusible messengers to the interaction between proteins on the ER and plasma membranes. In excitable cells, however, Ca^{2+} entry through VSCC is essential for refilling internal stores, but how the store content controls Ca^{2+} entry through these channels has not been clarified. To address this question, we will employ pituitary gonadotrophs as a cell model. Preliminary experiments indicate that $[Ca^{2+}]_i$ in these cells is necessary and sufficient to report the ER content and to stimulate Ca^{2+} entry as the ER content declines. In further experiments, we will combine experimental approaches and computations with a new mathematical model of the coupled oscillators. The store content in unstimulated and agonist-stimulated cells, the time-course and concentration dependence of GnRH-induced depletion of agonist-sensitive calcium pool, as well as the $[Ca^{2+}]_i$ and plasma membrane voltage measurements prior to, during, and after GnRH pulse will be examined and compared with theoretical predictions.

2. Identification of a Novel Calcium-Dependent Current in Pituitary Gonadotrophs. In agonist-stimulated gonadotrophs, Ca^{2+}-sensitive small K^+ channels (K_{Ca}) were considered to be the exclusive mediator of the Ca^{2+}-dependent plasma membrane conductance. However, in voltage-clamp experiments using the nystatin-perforated mode of the patch-clamp technique, we have recently observed that the reversal potential for the GnRH-activated whole-cell membrane oscillatory current is around -70 mV. This potential was more depolarized than the calculated equilibrium potential for K^+, which was -84 mV in our experimental conditions. Similarly, the residual oscillatory current observed after the addition of 1 μM apamin, a specific blocker of small K_{Ca}, had a reversal potential of -35 mV. We are planning to extend this investigation in GnRH-stimulated cells under similar conditions but with the replacement of all intrapipet K^+ by Cs^+. The reversal potential for the GnRH-activated current oscillations will also be examined in experiments in which the intrapipet Cl^- concentrations will be varied. In addition, a Ca^{2+}-dependent conductance will be analyzed in unstimulated cells in studies with Cs^+ in an intrapipet solution, following a tail current activated by Ca^{2+} entry through VSCC during depolarizing pulses. The ionic selectivity of such a tail current will be compared with the apamin-insensitive oscillatory current activated by GnRH. The Ca^{2+} permeability of this novel conductance and its role in spontaneous and GnRH-dependent electrical activity of gonadotrophs will also be addressed.

3. The Role of Mitochondria in Cellular Calcium Homeostasis in Gonadotrophs. A few recently published studies have suggested that the mitochondria have a role in cellular Ca^{2+} homeostasis in hepatocytes and oocytes. We are planning to study the role of mitochondria in intracellular calcium redistribution in gonadotrophs and lactotrophs. In these studies, three inhibitors of mitochondrial Ca^{2+} uptake, carbonyl cyanide m-chlorophenol-hydrazone (CCCP), carbonyl cyanide p-(trifluoro-methoxy)phenyl-hydrazone (FCCP), and rotenone, will be employed. Single Ca^{2+} measurements will be taken in identified gonadotrophs and lactotrophs bathed in a Ca^{2+}-deficient medium. Preliminary results show that the addition of these compounds is associated with an increase in $[Ca^{2+}]_i$. In contrast, none of the compounds was able to induce $[Ca^{2+}]_i$ responses in cells in which the ER Ca^{2+} pool was depleted by prior treatment with GnRH, thapsigargin (a blocker of the ER Ca^{2+} pump), or ionomycin. In additional experiments, we will examine the dose- and time-course of CCCP- and FCCP- action in gonadotrophs and lactotrophs, and compare the results with thapsigargin-induced rises in $[Ca^{2+}]_i$. Experiments with FCCP and CCCP will also be extended to streptolysin O-permeabilized gonadotrophs loaded with mag-fura 2. A major part of future investigations will be directed to the analysis of changes in plasma membrane and ER-oscillator activities in cells with altered mitochondrial uptake. To address the physiological significance of the redistribution of Ca^{2+} between the cytosol and mitochondria, the effects of FCCP, CCCP, and rotenone on gonadotropin and prolactin release will be analyzed.

4. Bidirectional Effects of Endothelins (ETs) on Prolactin Release. In addition to gonadotropins, ETs also stimulate prolactin and growth hormone release in perifused pituitary cells. However, in contrast to gonadotrophs and thyrotrophs, the transient stimulatory action of ET-1 is followed by the sustained inhibition

of prolactin release. While it has been shown that the stimulatory action of ET receptors on prolactin release is mediated by the phospholipase C-dependent pathway, the intracellular messenger pathway that mediates inhibitory action has not been characterized. To address this question, the effects of ETs on calcium signaling and prolactin release will be analyzed in pituitary cells from cycling female rats and GH_3 pituitary tumor cells. Initial experiments indicate that both cell types exhibit high and pulsatile basal prolactin release with a random frequency and amplitude, when perifused, and that such pulsatility was abolished in cells perifused with calcium-deficient medium. The addition of ET-1 caused a transient increase in prolactin release, which was followed by a rapid decrease to below the basal level, and the complete abolition of the pulsatile pattern of secretion. In cells bathed in calcium-deficient medium, only a transient spike phase of ET-1-induced prolactin release was observed, further suggesting that ET-1 inhibits exclusively extracellular calcium-dependent basal prolactin secretion. To clarify the receptor subtype involved in the inhibitory actions of ETs on prolactin release, the effects of ET-1 on the inhibition of prolactin release will be analyzed in the presence of BQ-123 and BQ-788, the antagonists of the ET_A and ET_B receptor subtypes, respectively. To evaluate plasma membrane channels involved in such calcium-dependent secretion, the effects of ET-1 on $[Ca^{2+}]_i$ will be examined in single lactotrophs and GH_3 cells under different experimental conditions. Furthermore, changes in the electrical activity of single cells in response to ET-1 will be investigated. Depending on the outcome of these data, the whole cell current and single channel activity may be analyzed in these cells. To ultimate goal of these experiments is to identify the plasma membrane channels involved in ET-1 mediated inhibition of prolactin release and the messenger(s) mediating the action of ETs on channel regulation.

5. Role of Voltage-Gated Calcium Entry on Phospholipase D Activation. It is well established that the excitability of GnRH neurons is essential for episodic neuropeptide release, but the mechanism by which electrical activity controls GnRH secretion has not been characterized. Our preliminary investigations indicate a possible role of phospholipase D in mediating the activity-dependent secretory pathways. For future investigations on this topic, immortalized GT1 neurons, which both secrete GnRH and express GnRH receptors, will be employed. In these cells, basal phospholipase D activity, estimated by the phosphatidylethanol assay, will be investigated in cells bathed in Ca^{2+}-containing and -deficient media, in the presence of calcium channel agonists and antagonists, as well as in high potassium-depolarized cells. The effects of activation of GnRH receptors on phosphatidylethanol, diacylglycerol, and cytidine diphosphate diacylglycerol production will also be examined. In addition, the dependence of phosphatidylethanol production on the phospholipase C pathway will be addressed. Both ethanol and propranolol, the latter being an inhibitor of phosphatidic acid-phosphohydrolase, will be employed to analyze changes in diacylglycerol production and GnRH release. The effects of these compounds on secretion will be analyzed in static cultures of GT1 cells and perifused cells.

Publications:

Krsmanovic LZ, Stojilkovic SS, Catt KJ: Pulsatile GnRH release and its regulation. Trends Endocrinol Met 1996; 7:56-59.

Kukuljan M, Vergara L, Stojilkovic SS: Modulation of the kinetics of $InsP_3$-induced $[Ca^{2+}]_i$ oscillations by calcium entry in pituitary gonadotrophs. Biophys J 1996 (in press).

Li Y-X, Keizer J, Stojilkovic SS, Rinzel J. Calcium excitability of the ER membrane: an explanation for IP_3-induced Ca^{2+} oscillations. Am J Physiol, Cell Physiol 1995; 38:C1079-1092.

Stojilkovic SS, Catt KJ. Expression and signal transduction pathways of endothelin receptors in neuroendocrine cells. Front Neuroendocrinol 1996; 17:327-369.

Stojilkovic SS, Catt KJ. Novel aspects of GnRH-induced intracellular signaling and secretion in pituitary gonadotrophs. J Neuroendocrinol 1995; 7:739-757.

Stojilkovic SS, Catt KJ. Receptors and secretory actions of endothelins in neuroendocrine cells. Front Endocrinol 1995; 15: 135-144.

Stojilkovic SS, Zheng L, Tomic M, Cesnjaj M, Jobin RM, Catt KJ. GnRH-induced intracellular signaling in pituitary gonadotrophs. The Second International Symposium on Fertility Regulation 1995 (in press).

Stojilkovic SS. Endothelin receptors and gonadal function. Eur J Endocrinol 1996 (in press).

Stojilkovic SS. Measurement and manipulation of intracellular ions. Endocrine News 1996 (in press).

Stojilkovic SS. Calcium signaling in pituitary cells. Trends Endocrinol Metab 1996 (in press).

Tomic M, Stojilkovic SS. GnRH-induced calcium and current oscillations in gonadotrophs. Trends Endocrinol Metab 1996 (in press).

Tomic M, Jobin RM, Vergara LA, Stojilkovic SS. Expression of purinergic P_2 channels and their role in calcium signaling and hormone release in pituitary gonadotrophs. J Biol Chem 1996 (in press).

HDB

HERITABLE DISORDERS BRANCH

Joan C. Marini, M.D., Ph.D., Chief

Members of the Heritable Disorders Branch perform both clinical and basic research into the etiology, diagnosis, and therapy of human genetic diseases. Clinically, inborn errors of metabolism and connective tissue disorders in children are especially emphasized. Clinical investigators admit patients to the pediatric ward of the NIH Clinical Center under protocols to study rare diseases. These disorders are chosen both for the benefit that can be offered to affected patients, and because identifying the cause of a specific dysfunction often unveils a normal process. All the clinical members of the Branch also conduct basic research on their diseases of interest in addition to providing clinical care. The basic research groups in the Branch investigate molecular or biochemical aspects of human genetic disease. The basic groups interact closely with their clinical counterparts on a technical level.

CONNECTIVE TISSUE DISORDERS

The Section on Connective Tissue Disorders, directed by ***Joan C. Marini***, conducts a fully integrated program of laboratory and clinical research, aimed at characterizing the molecular mechanisms of the heritable connective tissue disorders osteogenesis imperfecta (OI) and Ehlers-Danlos Syndrome, and at applying this information to the diagnosis and treatment of affected patients.

MOLECULAR THERAPEUTICS FOR OSTEOGENESIS IMPERFECTA. OI is a dominant disorders of connective tissue, in which affected individuals have fragile bones susceptible to fracture from minimal trauma. OI is caused by mutations in type I collagen, the most abundant structural protein in the body. In general, mutant chains are incorporated into the collagen helix, secreted from the cell and integrated into the intracellular matrix. Thus, a gene therapy approach aimed at adding a normal collagen gene would not be effective in such a dominant negative disorder. A major interest of this Section is to develop selective antisense suppression of the mutant collagen allele, as an approach to therapeutic intervention. Recently, these workers have focused on antisense hammerhead ribozymes to increase both specificity and efficiency of antisense suppression. They have demonstrated the allele-specificity of the hammerhead ribozymes *in vitro*. They selected two unrelated patients whose naturally occurring mutations are responsible for the formation of a new ribozyme cleavage site in the mutant allele. In one case, the mutant allele contained a GUC cleavage site; in the second case, the mutation created two new cleavage site, GUU and UUC. For each patient, a cDNA fragment from both normal and mutant alleles was cloned into pGEM vector and mRNA was generated by *in vitro* transcription.

Increasing amounts of ribozyme, ranging from a RZ/substrate ratio of 1:1 to 1:125, were incubated with the synthetic transcripts, products were separated on denaturing gels and quantitated by densitometry or direct counting. In all cases, the ribozyme was absolutely specific for the mutant allele. This is the first demonstration that ribozymes selectively cleave mRNA molecules carrying a naturally occurring point mutation causing a genetic disorder. In all cases, the cleavage activity depended on the time and temperature of incubation, as well as on the ratio of ribozyme to substrate. For the GUU and UUC cleavage sites, all experiments were also performed using synthetic RNA target of progressively increasing length, from about 500 to 1000 nt. The increasing substrate length did not significantly affect the rate of ribozyme cleavage. This feature is encouraging for the *in vivo* activity of ribozymes, involving cleavage of full length mRNA.

The Section also studied the rate of cleavage by ribozymes when the target synthetic RNA was mixed with different amounts of non-specific RNA or normal allele RNA. The rate of cleavage by ribozymes was not affected by the presence of total RNA up to a 500:1 weight ratio of total RNA to mutant target. The rate of cleavage is decreased approximately 50% by the presence of normal allele synthetic target at a ratio of 1:1 mutant/normal substrate. This demonstrates that binding of the complementary arms to the non-cleavable target has a slow release time *in vitro*. The binding can be destabilized by introducing a single mismatch into the middle of one of the complementary arms of the ribozyme. Under these conditions, even a 16-fold molar excess of synthetic non-cleavable substrate does not decrease the RZ cleavage rate. The group is developing vector-mediated transfections for new experiments testing ribozyme effectiveness in cultured cells.

DEVELOPMENT OF A NON-LETHAL MURINE MODEL OF OI. Currently, no animal model for OI is available that is non-lethal, has significant physical manifestations and expresses a mutation similar to a naturally occurring collagen mutation. In this Section, Antonella Forlino has initiated a project, in cooperation with the LMGD, NICHD, to generate a conditional non-lethal murine model for OI. By site-directed mutagenesis on a subcloned murine COL1A1 fragment, she changed a G → T in exon 23 at nt 1546. This mutation was previously characterized in this Section in a case of human type IV OI and causes the substitution of gly349 → cys. A C → T transition at nt 1566 and a C → G transversion at nt 1568 were also generated to change Leu356 → Met to enable the detection of the mutant protein by CNBr digestion. In addition, she generated a GUC cleavage site for hammerhead ribozyme cleavage of the mutant allele mRNA by changing a C → G at nt 1551.

Since the severe OI phenotype could compromise the reproductive success of the affected mice, they plan to suppress the expression of the mutated allele in the first generation. To do this, a lox-stop-lox transcription termination cassette has been inserted into intron 22, which is located upstream of the exon containing the disease-causing mutation. The linearized construct was electroporated into embryonic stem cells derived from the 129Sv murine strain. Recombinant clones were selected using a double selection with neomycin and gancyclovir. Three recombinant ES clones were microinjected into recipient C57BL/6 blastocysts, which were then inserted into foster mothers. From this first round of microinjection, they have obtained two male mice which appear to be chimeras on the basis of coat color.

MOLECULAR CHARACTERIZATION OF OI BONE. This Section investigates the molecular and biochemical characteristics of cultured OI osteoblasts in order to gain insight into the underlying pathobiology of this bone disease. They have electrophoretically analyzed the type I collagen synthesized by osteoblasts and fibroblasts in 10 patients affected by type IV OI. Most osteoblast collagens displayed a dramatic shift of the electrophoretic baseline of the collagen chain or a broader $\alpha1(I)$ band, as compared with the matching fibroblast sample. Using cells from a patient with a novel $\alpha1(I)$ gly 352 → cys substitution, the electrophoretic abnormality was demonstrated to be post-translational in origin when fibroblast and osteoblast collagen synthesized in the presence of α,α'-dipyridyl co-migrated. These data suggest that cellular processing of mutant collagen protein differs between OI fibroblasts and osteoblasts.

They have also investigated the response of OI osteoblasts to TGF-β stimulation, treating the osteoblasts from an OI proband with an $\alpha1(I)$ Gly222→Ala substitution and an age-matched control. The mRNAs for both of the type I collagen chains were up-regulated by 4-48 hrs of stimulation. Collagen stimulation in the OI osteoblasts was approximately half that obtained in control cells. The most dramatic changes were detected in the expression of the proteoglycans decorin and biglycan. In cells treated with 5 ng/ml TGF-β, decorin mRNA was upregulated 4-fold in control OB. In OI OB, only a transient increase was detected, which was significantly less than the control response. Biglycan was up-regulated more than 6-fold in control OB and less than 2-fold in OI OB. These data suggest that OI OB may be generally less responsive to TGF-β signalling and may indicated abnormal function at the cell-matrix interface.

MUTATIONS IN OI PATIENTS. The Section continues to identify mutations that are responsible for OI. The group had previously identified a unique deletion in one allele of the $\alpha1(I)$ gene in a family with dominant type III OI, extending from the last three nucleotides of exon 33/34 to the middle of intron 36. They have now demonstrated that there are three alternatively spliced forms of the mRNA from this allele. One form (40%) is out-of-frame and leads to a partial null allele, the others (60% total) are in-frame. Apparently, the effect of the partial null allele moderates the expected lethal phenotype caused by the large deletion.

In a patient with lethal type II OI, the Section has identified a gly 421 → asp substitution which generates unusual electrophoretic mobility. Even without post-translational modification, the chains have unusually slow migration, suggesting the presence of a kink in the chains. The location of this mutation in the $\alpha2(I)$ chain is also supportive of the Regional Model previously proposed by this group to correlate genotype and phenotype in OI,

CLINICAL INVESTIGATIONS OF OI. This Section has continued its investigation of the clinical aspects of OI, especially growth deficiency, neurological abnormalities and physical rehabilitation. The Growth Hormone treatment trial is in its final year. At the conclusion of the study, 26 type III and IV OI children will have been treated for at least one year. From the first 20 children treated, nine responded with a sustained increase in linear growth rate; the responder group was composed almost entirely of children with type IV OI. This study has also provided interesting data on the responses of bone synthetic peptides to growth hormone stimulation. The full patient group shows a stimulation of IGF-I and IGF-BP3, as well as in collagen terminal peptide. With increasing time of treatment, the stimulation of these peptides in the growth responder group exceeded that in the non-responders. This suggests that there may be a relative insensitivity to growth hormone in OI bone and that the signaling defect can be more easily overcome in the responder group. The responder group also experienced significant positive changes in bone morphometric parameters after one year of treatment.

The extent to which basilar invagination was a feature of OI was first appreciated by this clinical program. Fifteen NIH OI patients have been determined to have BI. A scanning protocol has been established to determine the natural history of BI and the optimum nature and timing of the surgical response.

INVESTIGATIONS OF EHLERS-DANLOS SYNDROME. Ehlers-Danlos Syndrome (EDS) is a connective tissue disorder whose symptoms overlap with those of OI but do not include brittle bones. The Section is investigating a number of cases of EDS IV, a severe form of the disorder characterized by vascular rupture, and frequently associated with abnormalities of type III collagen. Marini's group is pursing the molecular defect in two cases with identified protein abnormalities, one a quantitative reduction of type III collagen, and the other a qualitative abnormality of electrophoretic migration.

GLYCOGEN STORAGE DISEASE

The Section on Cellular Differentiation, led by ***Janice Chou***, seeks to understand the molecular genetics of glycogen storage disease type 1 (GSD-1) and methionine adenosyltransferase (MAT) I/III deficiency, to characterize the physiological functions of pregnancy-specific glycoprotein (PSG) and to study the molecular mechanisms regulating differential alkaline phosphatase (AP) expression in the human placenta. The Section has established the molecular basis of GSD-1a and developed an non-invasive gene-based diagnosis of this disorder. This research group has also generated *G6Pase*-deficient mice that mimic the pathophysiology of human GSD-1a patients. The knockout mouse provides a valid animal model to study the pathogenesis of GSD-1a, to delineate the mechanisms of G6Pase catalysis, and to develop future therapeutic approaches. The Section has established the molecular basis of MAT I/III deficiency, developed a DNA-based diagnosis for this disorder, and demonstrated that a complete lack of MAT I/III activity leads to neurological abnormalities. The research group has also increased our understanding of the roles of PSGs during pregnancy and characterized cis-acting elements and transcription factors that control differential AP expression during malignant transformation of the human placenta.

Glycogen Storage Disease Type 1. GSD-1 is caused by a deficiency of glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis. This metabolic disease is characterized by hypoglycemia, hepatomegaly, growth retardation, enlarged kidneys, hyperlipidemia, hyperuricemia, lactic acidemia, and in adults, a high incidence of renal failure and hepatic adenomas. Diagnosis of GSD-1a currently is established by demonstrating the lack of G6Pase activity in the patient's biopsied liver specimen. Recent cloning of the G6Pase gene and identification by Dr. Chou's group of mutations within the gene that cause GSD-1a allowed for the development of a DNA-based diagnostic method. Using single strand conformation polymorphism analysis (SSCP) and DNA sequencing, this group has characterized the *G6Pase* gene of 70 unrelated patients with enzymatically confirmed diagnosis of GSD-1a and detected mutations in all except 17 alleles (88%). Sixteen mutations were uncovered which were shown by expression to abolish or greatly reduce G6Pase activity and are therefore responsible for the GSD-1a disorder. R83C and Q347X are the most prevalent mutations found in Caucasians, 130X and R83C in Hispanics, and R83H in Chinese. The Q347X mutation has thus far been identified only in Caucasian patients, and the 130X mutation only in Hispanic patients. Their results demonstrate that the DNA-based analysis can accurately, rapidly, and non-invasively detect the majority of mutations in GSD-1a. This DNA-based diagnosis now permits prenatal diagnosis among at-risk patients and serves as a data-base in screening and counseling patients clinically suspected of having this disease.

At present, treatments of GSD-1a focus solely on controlling symptomatic hypoglycemia. Patients who follow careful metabolic control continue to have blood lactate, cholesterol, and triglyceride values above the normal range and kidney function eventually becomes compromised. It is not clear whether long-term complications including gout, hepatic adenomas, and osteoporosis will eventually develop in the patients despite good dietary intervention. To understand the pathogenesis of this disorder, to delineate the mechanisms of G6Pase catalysis, and to develop future therapeutic approaches, Dr. Chou's group has generated *G6Pase*-deficient mouse strains by gene targeting and shown that the G6Pase$^{-/-}$ mice manifest essentially the same pathophysiology as human GSD-1a patients, including hypoglycemia, growth retardation, hepatomegaly, kidney enlargement, hyperlipidemia, and hyperuricemia. Human GSD-1a patients who do not receive dietary treatment die early, usually in the teens, of liver and/or kidney complications. Histopathological examinations of the G6Pase$^{-/-}$ animals revealed marked glycogen storage in the liver and kidney, and fat deposition in hepatocytes. The G6Pase$^{-/-}$ mice survived an average of 4 to 5 weeks and necropsy analysis revealed evidence of severe liver and renal lesions. The extensive similarities between homozygous mutant animals and human GSD-1a patients indicate that the G6Pase$^{-/-}$ mouse is a valid animal model for the study of GSD-1a.

This well-defined mouse model of GSD-1a provides an opportunity to examine the two theories proposed for G6Pase catalysis. The multi-functional conformation-substrate-transport model is supported by observations that G6P transport and hydrolysis appear synchronous. The multi-component translocase-catalytic unit model, however, is supported by observations that liver microsomes in GSD-1b and 1c patients have G6Pase activity but no substrate or product transport activity. To assess these two models, Dr. Chou's group has examined microsomal G6P transport and hydrolysis in liver and kidney microsomes from G6Pase$^{+/+}$ and G6Pase$^{-/-}$ mice. They showed that the knockout of the *G6Pase* gene destroys both G6Pase activity and G6P transport, providing direct evidence of the tight coupling between G6P transport and G6Pase activity. By examining G6Pase in the liver and kidney, the primary gluconeogenic tissues, Dr. Chou and coworkers demonstrated that G6P transport and hydrolysis are performed by separate proteins. This research group proposed a modified translocase-catalytic unit model, in which G6Pase catalysis consists of two tightly coupled reactions, G6P transport and G6P hydrolysis. Inactivation of transport prevents substrate access to the hydrolytic center while inactivation of hydrolysis abolishes transport by uncoupling an essential link.

G6Pase is primarily expressed in the liver and kidney and liver play a major role in glucose homeostasis. In order to gain insight into the molecular mechanisms regulating liver-specific expression of the human *G6Pase* gene, Dr. Chou's group characterized *G6Pase* promoter activity in hepatic and nonhepatic cells. Transient expression assays demonstrated that the *G6Pase* promoter is active in hepatoma cells but not in choriocarcinoma or 3T3 cells. Nucleotides -234 to +3 DNA region upstream of the transcription start site of the *G6Pase* gene directed optimal *G6Pase* expression in hepatoma cells. Sequence analysis indicated the presence of multiple transcription factor binding sites within this region, including motifs for liver-enriched factors such as hepatocyte nuclear factor (HNF) 1 (-226/-212), HNF3 (-180/-174, -139/-133, -91/-85, and -81/-75), HNF4 (-240/-229 and -231/-220), and C/EBP (-130/-125). Deletion analysis revealed the presence of three activation elements at nucleotides -234/-212, -181/-125, and -124/-71 of the *G6Pase* promoter. Gel mobility shift assays showed that HNF1, HNF3γ, and C/EBPβ bind to their respective cognate sites. Cotransfection studies demonstrated that HNF1α (not HNF1β) and HNF3γ transactivate *G6Pase* expression, while C/EBPβ plays little role in its expression. The results indicate that liver-specific expression of the *G6Pase* gene is positively controlled by liver-enriched transcription factors, HNF1α and HNF3γ.

Methionine Adenosyltransferase I/III Deficiency. Methionine adenosyltransferase (MAT) is a key enzyme in transmethylation, transsulfuration and the biosynthesis of polyamines. Three forms of MAT (I, II, and III) have been identified in mammalian tissues. MAT I and III, which are encoded by the single copy *MAT1A* gene, represent tetramers and dimers formed from identical α1 subunits and are synthesized primarily in the liver. Genetic deficiency of MAT I/III causes isolated persistent hypermethioninemia and, in some cases, unusual breath odor or neural demyelination. However, the molecular mechanism(s) underlying this deficiency has not been clearly defined. To study the molecular genetics of this disorder, Dr. Chou's group characterized the human *MAT1A* gene and showed that it is a single copy gene consisting of 9 exons and 8 introns. Using SSCP and DNA sequence analyses, Dr. Chou's group characterized the *MAT1A* gene in eleven individuals with isolated persistent hypermethioninemia, including two with brain demyelination. Mutations that abolish or reduce MAT activity were detected in the *MAT1A* gene of all eleven individuals, thus establishing the molecular basis of MAT I/III deficiency. Both patients with demyelination are homozygous for mutations that alter the reading frame of the encoded protein such that the predicted MATα1 subunits are truncated and enzymatically inactive. The product of MAT, adenosylmethionine (AdoMet), is the major methyl donor for a large number of biologically important compounds including the two major myelin phospholipids, phosphatidylcholine and sphingomyelin. Both are synthesized primarily in the liver. Their findings demonstrate that isolated persistent hypermethioninemia is a marker of MAT I/III deficiency, and that complete lack of MAT I/III activity can lead to neurological abnormalities. Therefore, a DNA-based diagnosis should be performed for individuals with isolated hypermethioninemia to assess if therapy aimed at the prevention of neurological manifestations is warranted.

Analyses of the *MATA1* mutations in these eleven MAT I/III deficient individuals and the available parents indicate that these mutations are inherited as autosomal recessive traits. However, dominant inheritance of hypermethioninemia, also hypothesized to be caused by MAT I/III deficiency, has been reported in two families. Dr. Chou's group has characterized the *MATA1* genes in all available members of the two families and uncovered a single G→A mutation at nucleotide 791 in one *MATA1* allele that converts an Arg at position 264 to a His (R264H). Substitution of Arg-264 with His (R264H, the naturally occurring mutant), Leu (R264L), Asp (R264D), or Glu (R264E) greatly reduced MAT activity and severely impaired the ability of the MATα1 subunits to form homodimers essential for catalytic activity. On the other hand, when Lys was substituted for Arg-264 (R264K), the mutant α1 subunit was able to form dimers that retains high MAT activity, suggesting that residue 264 is involved in the formation of inter-subunit salt bridge. They further demonstrate that the R264H mutation exerts a dominant effect by forming enzymatically inactive R264/R264H MATα1 heterodimers.

Pregnancy-Specific Glycoproteins and Alkaline Phosphatases. National statistics reveal that over 50% of pregnancies end in miscarriages or abortion. Moreover, expression of many pregnancy-associated proteins, including PSGs and APs, is altered in complicated pregnancies and human tumors. Therefore, understanding the role of these proteins in implantation, maintenance of pregnancy, and fetal development is fundamental to women's health. With this in mind, Dr. Chou's group has focused on PSGs and APs. PSGs form a large family of essential pregnancy proteins, but their biological function is unknown. Many PSG members contain an RGD motif within the N-domain. In other proteins, this motif has been implicated in receptor ligand interactions. This research group has investigated whether one function of the RGD-containing PSG is to interact with cells of the maternal immune system. Purified placental PSG bind to normal human peripheral blood mononuclear cells, activated with phorbol ester. This binding activity can be mimicked using a chemically synthesized peptide ligand containing the RGD motif in PSG11s. The PSG11s receptors are present on cells of the myeloid cell lineage but not of the T-cell or B-cell lineages. The consequences of this binding interaction altered the cytokine secretion profiles of monocytes.

Human placentas express high levels of the placental AP (*PLAP*) gene and low levels of a highly related gene, germ cell AP (*GCAP*). Malignant transformation of the placenta is accompanied by a reversal of this pattern of expression. In earlier studies, Dr. Chou's group showed that three Sp1-binding GC-rich DNA elements (sites I, II, and III) in the 5'-flanking region of the *GCAP* gene directed optimal *GCAP* gene expression in choriocarcinoma cells. They now show that the first 100 base pairs upstream of the *GCAP* gene, which contains sites I and II, constitutes a minimal *GCAP* promoter. The simultaneous presence of both sites I and II is necessary for *GCAP* expression and its induction by sodium butyrate. The *PLAP* promoter directs only a very low level of gene expression in choriocarcinoma cells; the expression does not respond to butyrate. The -100/-1 DNA regions between the *GCAP* and *PLAP* promoters differ by only 8 base pairs. However, the GC-rich stretches in sites I and II of the *GCAP* promoter are disrupted in the corresponding *PLAP* promoter. This disruption blocks or markedly reduces the binding of choriocarcinoma nuclear factors to the *PLAP* promoter, leading to a reduction in expression and a loss of butyrate response. Nnucleotides -75 to -58 in both AP promoters, which bind a human Y-box binding protein, appear to downregulate *GCAP* expression.

INBORN ERRORS OF METABOLISM

The Section on Human Biochemical Genetics, under the direction of ***William Gahl***, investigates human inborn errors of metabolism which may help elucidate biochemical pathways and cell biological phenomena.

The Search for the Cystinosis Gene. For the past 15 years, members of the Section have characterized nephropathic cystinosis both clinically and biochemically, describing the myriad complications of the disease and demonstrating the efficacy of therapy with the cystine-depleting agent, cysteamine. However, neither the gene for this autosomal recessive disease nor its protein product, the lysosomal cystine transporter, has been isolated. Previously, Dr. Geraldine McDowell of the Section spearheaded an international collaborative effort which made use of linkage analysis to map the cystinosis gene to a 3.1 cM region of chromosome 17p13 flanked by the microsatellite markers D17S1828 and D17S1798. In collaboration with Drs. David Ledbetter and Akira Tanigami of the NCHGR, Dr. McDowell then created a yeast artificial chromosome (YAC) contig between these markers and reported an integrated genetic and physical map of the region. Using one pertinent YAC clone, 898A10, Dr. Takao Isogai identified new polymorphic microsatellite repeat markers in the cystinosis gene region. One such marker, D17S2167, was localized by radiation hybrid analysis to within 10.2 cR_{8000} of D17S1828. (Radiation hybrid mapping involves the use of a panel of rodent/human hybrids in which the human DNA has been cleaved by x-ray exposure. Markers close to each other are less likely to have a break between them and, therefore, will be lost or retained together upon fusion with rodent cells. Distant markers are more likely to have a break between them and will be lost or retained independently. The presence of pertinent markers in the cystinosis gene area on the 83 hybrids of the Stanford G3 radiation hybrid panel was determined by PCR assay. The order and relative distances of these markers were determined by multipoint analysis using the program RHMAX4K.) Haplotype analysis in two separate informative families revealed recombination events which placed the cystinosis gene between two markers, D17S1828 and D17S2167.

Using these markers, three P1 clones in the region of the cystinosis gene were isolated, each containing approximately 70 kb of human DNA. Two of these P1 clones overlapped, since they shared nonpolymorphic markers and yielded some identically sized fragments on restriction endonuclease digestion with *EcoR1* and *HindIII*. Therefore, the two P1 clones comprise a contig across the region of interest, meaning that one of the P1 clones must contain the cystinosis gene. In addition, the area of interest was verified by pulsed-field gel electrophoresis of the P1 clones to be approximately 130 kb in size.

The P1 DNA is now being used in a variety of gene-seeking strategies, including screening a cDNA library, 3'-exon trapping, and shotgun sequencing of libraries created from each P1 clone, followed by searching of computer data banks for corresponding anonymous expressed sequence tags. Candidate genes will be used to probe northern blots containing RNA from several cystinosis patients. RT-PCR will also be performed using patients' RNA. The ultimate cloning of the cystinosis gene should eventually reveal how a functional lysosomal transport protein is synthesized, targetted, processed, and integrated into the lysosomal membrane.

Pigment Disorders. The Section continues to study mammalian pigmentation from both basic research and disease-oriented standpoints. A primary issue in melanogenesis involves the entry of tyrosine, the initial substrate precursor of the pigment melanin, into the pigment-producing vesicle, or melanosome. Gahl's group, using cultured melan-a murine melanocytes, has demonstrated that this process is carrier-mediated. The tyrosine and neutral amino acid transporter displays counter-transport, saturability, and stereospecificity, and resembles lysosomal system h of rat thyroid FRTL-5 cells in its substrate specificity. The existence of this carrier system explains how melanosomes are able to synthesize melanin, since tyrosine provides the substrate precursor for this critical pigment.

Another mystery of the melanocyte is how its specific subcellular organelles, i.e., melanosomes, are formed. This may involve the function of rabs, a family of nontransforming monomeric GTP-binding proteins in the ras superfamily. Similar to ras, these proteins have four highly conserved regions (designated I to IV) involved in GTP binding and hydrolysis. They also contain a consensus carboxy-terminal amino acid sequence containing two cysteine residues. Geranyl-geranylation of a carboxy-terminal cysteine allows for anchoring of the rab to the plasma membrane or to a vesicular membrane. Subsequently, in a fashion not fully elucidated, rabs regulate membrane trafficking and vesicular fusion and targeting. In melanocytes, rabs may be critical for pigment production, since this function is performed by the melanosomal vesicle. Therefore, members of the Section have catalogued the melanocyte's rab proteins using a PCR-based strategy to isolate partial rab cDNA sequences from pigmented human melanoma cells. Degenerate PCR primers were designed based on the amino acid sequences WDTAQ and GNKXD, two GTP-binding domains that are strictly conserved in all members of the human rab family. The distances between these two domains are also nearly invariable.

Twenty-four different small GTP-binding proteins were identified in two rounds of screening; eight of these (rap1A, rab11, rab1A, ras-like protein, R-ras, rab13, rho, and LLrep3) accounted for approximately 85% of the GTP-binding protein expression of the melanoma cells. Two other rab genes were isolated and given names designating their novel status. One, rab30, codes for a protein of 203 amino acids with minimal homology to previously documented GTPases. Another, rab22b, appears to be an isoform of the human homologue of canine rab22. The complete cDNA sequences of these genes were determined by a combination of 3'-RACE (rapid amplification of cDNA ends), 5'-RACE, and cDNA library screening. On northern blot analysis, both rab30 and rab22b mRNA displayed a nearly ubiquitous pattern of expression across the range of tissues examined. Rab22b and rab30 were mapped to chromosomes 18 and 11, respectively.

Finally, the complete cDNAs of two rab isoforms, rab27a and rab27b, were isolated from human melanoma cells and melanocytes. Rab27a is the human homologue of a rat megakaryocyte rab called ram p25. Rab27b corresponds to a small GTP-binding protein, c25KG, which was previously purified from platelets but whose cDNA had not been cloned. Sequence comparisons with known rabs indicate that rab27a and rab27b comprise a melanocyte/platelet subfamily within the rab family. Their expression in both melanocytes and platelets makes rab27a and rab27b candidates for involvement in mouse and human mutations causing the combination of pigment dilution and a platelet storage pool defect. Murine examples include *pale ear*, whose phenotypic and genetic counterpart in humans is Hermansky-Pudlak syndrome (HPS). HPS consists of the triad of tyrosinase-positive albinism, a storage pool deficiency due to absent platelet dense bodies, and accumulation of ceroid lipofuscin in cellular lysosomes. Although the basic defect is unknown, each of these abnormalities could be related to a vesicular membrane defect. In collaboration with Dr. Ray Boissy of the Medical College of Cincinnati, the expression of rab27a and rab27b in HPS cultured melanocytes is being examined by northern blot analysis and by RT-PCR.

The Section's interest in HPS also extends to the clinical arena. In August of 1995, a protocol was established to investigate this autosomal recessive disorder common in Puerto Rico. The goals are to determine the basic defect, better characterize the disease among Puerto Rican and non-Puerto Rican patients, and develop therapy, particularly for the pulmonary fibrosis which generally proves fatal in the fifth decade of life. Over 30 patients age 3 to 60 years of age, half of Puerto Rican ancestry, have been studied at the NIH Clinical Center. Insights into the basic defect have followed the lead that renal tubular epithelial cells sloughed into the urine of HPS patients contain dolichols, hydrophobic lipids composed of chains of isoprene units. Dolichols are offshoots of the cholesterol synthetic pathway, and several children with HPS have displayed high cholesterol levels. Many patients also exhibit elevated blood concentrations of coenzyme Q_{10}, another product of the cholesterol synthetic pathway. Finally, collaboration with Dr. Josh Zimmerberg's group has revealed an increased fusability of HPS red blood cell membranes, consistent with an increased content of highly hydrophobic lipids such as dolichols. All this circumstantial evidence prompts further pursuit of the hypothesis that the basic defect in HPS involves up-regulation of the cholesterol synthetic pathway.

The clinical characterization of HPS includes extensive blood and urine chemistries as well as specialty consultations. As a group, the HPS patients have exhibited normal renal, hepatic, and endocrine function. As expected, platelet aggregation studies have been abnormal, but lymphocyte phenotyping results appear within the normal range. Visual acuity ranges from 20/100 to 20/400, with variable degrees of nystagmus and iris transillumination. Granulomatous colitis has been a prominent feature in only 10% of patients. Dermatologic examination has revealed extremely variable degrees of pigmentation, increased hirsutism, and prominent nevi in several patients. Pulmonary function tests have been abnormal in individuals as young as 12 years old, and show a restrictive pattern in the majority of patients over 30 years of age. Some patients have characteristically thickened bronchial septa on high-resolution CT scans of the lung. Pulmonary lavage performed on 5 adults with HPS has shown a 5-10 fold increase over normal in the number of cells recovered. Further analysis of the inflammatory mediators produced by these cells could indicate the type of therapy to which HPS patients will respond.

Lysosomol Cystine Transporter. The Unit on Genetic Diseases Involving Cystine Storage is directed by ***Jean Butler***. Commercially obtained cystine binding protein of E. coli has been purified by HPLC, yielding a pure cystine binding protein not similar to any protein in the data banks. This protein has been partially sequenced, an N-terminal peptide made and an antibody to this peptide obtained which reacts with the whole protein. Cystine binding protein of E. coli is being cloned by making oligonucleotides of sections of the known N-terminal sequence of cystine binding protein and using them as primers to identify this protein in the E. coli library. By this method, we have obtained 260 bp of sequence of the E. coli DNA of cystine binding protein.

Niemann-Pick C Disease. Niemann-Pick C disease was shown to be reversibly induced in normal fibroblasts by hydrophobic amines and certain steroids, thus creating a model system for study of this disease. We are identifying the trafficking patterns and metabolism of intracellular cholesterol using various inhibitors and stimulators.

Ascorbic Acid Transporter. We have shown that normal and cystinotic fibroblasts take up ascorbic acid by two mechanisms, a high affinity Na^+ dependent transporter and a low affinity Na^+ independent transporter, neither of which can be accounted for by diffusion. Transport of ascorbic acid by the low affinity transporter is not different from normal, however transport in cystinotic cells by the high affinity transporter is about 40% of that seen in normal cells. The Km's for both types of cells were the same while the Vmax of the high affinity transporter was lower than normal for cystinotic cells.

DRUG DETOXIFYING ENZYMES

The Setion on Genetic Disorders of Drug Metabolism, under the direction of ***Ida S. Owens***, studies the regulation of the UDP glucuronosyltransferase (transferase) gene family at the molecular level using the human system. Several transferase isozymes catalyze critical substrates such as bilirubin, bile acids, steroids, natural food components, food additives, and exogenous toxins; the family of glucuronidating (detoxifying) enzymes is present in the liver, as well as nonhepatic tissues. The aim of Owens' program is to isolate and characterize the genes and cDNAs encoding these isozymes.

Extension of the UGT1 Locus. Dr. Owens' group has essentially completed the description of the *UGT1* locus. Its description has been critical to determining defects in patients with Crigler-Najjar(CN) diseases. The original version of the gene complex, *UGT1A-UGT1F* and recently renamed *UGT1A1-UGT1A6*, codes for 1 bilirubin, 3 bilirubin-like, and 1 phenol UDPglucuronosyltransferases. In the 5' region of the locus, 6 different exons 1 (including 1 pseudo gene) each with an upstream promoter and each encoding the amino terminus of an isoform, are arrayed in series with 4 common exons encoding 5 identical carboxyl termini in the 3' region. The most 5' exon, *UGT1A6*, of the original version of the locus was used to select other cosmid clones to extend the locus by more than 310 kb. Dr. Owens' group identified, sequenced, and mapped 6 additional exons 1 coding for *UGT1A7-UGT1A12P* genes. The total size of the locus is more than 410 kb: 95 kb for the original version and a minimal of 350 kb for the extended portion.

Nomenclature-- The UGT1 Locus. The novelty of this locus created confusion among the Human Nomenclature Committee and others as to an appropriate designation: a single gene or multiple genes. An agreement has been reached, consistent with the designation by Dr. Owens' group of the original locus, that the locus represents multiple genes of the *UGT1* family of transferase isozymes with each having independent regulation but linked through the 3' end. To be consistent with the nomenclature for other transferase families and in agreement with other researchers in the field, Dr. Owens' laboratory took the lead in renaming the genes in the *UGT1* complex. The previous designation of the genes *UGT1A* through *UGT1F* will be renamed in this document and in future publications: *UGT1A1* through *UGT1A6*. In the extended portion of the locus, newly identified exons 1 representing genes, will continue with the nomenclature: *UGT1A7* through *UGT1A12P*. Mutant alleles for the bilirubin transferase will be designated *UGT1A1*1, UGT1A1*2*, etc. based on the order of discovery of the alterations in the exon 1 of the *UGT1A1* gene. Alleles with mutations in a common exon will be designated *UGT1*1, UGT1*2* etc. demonstrating an effect on the entire *UGT1* locus.

UGT1A7 through *UGT1A12* is a cluster of exons 1 with 86 to 91% sequence identity which represents a subfamily within the *UGT1* family. Due to at least 6 nucleotide deletions scattered through out the most 5' exons 1, the two are nonviable and are pseudo: *UGT1A11P* and *UGT1A12P*. Hence, only 4 of the last 6 exons 1 are predicted to encode a viable mRNA. Except for *UGT1A12P*, a TATA box element is located between positions -137 to -121 bp upstream of each exon 1 in this cluster. The element for *UGT1A7* through *UGT1A10* is: AAT_9AAA or $AT_{14}AT$ and that for *UGT1A11P* is AT_8G_6T. Based on nucleotide alignment of the highly similar upstream regions of the *UGT1A7* through *UGT1A11P* genes, nucleotides from -40 to -178 bp upstream of exon 1 of *UGT1A12P* have been deleted.

Tissue Distribution of Messenger RNA for the Newly Uncovered Genes. Dr. Owens' group, using exons 1-specific probes, showed that mRNA encoding *UGT1A7* through *UGT1A10* is located primarily in the colon, small intestine, bladder, skeletal muscle, and heart. The *UGT1A10* gene is expressed only in heart muscle. Only *UGT1A9* is also distributed in the typical glucuronidating organs, liver and kidney.

Synthesis of cDNAS encoding Newly Uncovered Genes and Substrate Specificity of the Isoforms. In order to expedite the determination of substrate specificity of these newly uncovered and encoded isoforms, Dr. Owens' group constructed the equivalent cDNAS for expression in COS-1 cells. Since a cDNA, HLUG P4, encoded by *UGT1A9* had already been reported in the literature, they isolated the same cDNA from a hepatocyte cDNAλZAP library to use in the construction of cDNAS representing the *UGT1A7*, *UGT1A8* and *UGT1A10* genes. Overlaps between the exon 1 of a gene and a common fragment from the UGT1A9DNA was made by using polymerase hain reaction-amplified material. The overlap was extended by the Klenow fragment to generate the unique 5' terminus and a portion of the common end of the new cDNA. This strategy was successful because the 3' end of exon 1 of *UGT1A7-UGT1A10* genes contains a common 60 nucleotide sequence (the overlap) that is contiguous with the identical 3' common end (738 nt) for all genes encoded at this locus. Hence, the 798 bp-3' end of the *UGT1A7* through *UGT1A10* cDNAS is predicted to be identical. The constructions of the cDNAS were required since Dr. Owens and her group were unable to select these particular cDNAS from a human hepatocyte cDNAλZAP library. Two cDNAs representing exons 1 of *UGT1A3* and *UGT1A5* uncovered during the initial characterization of the *UGT1* locus have been constructed in a similar fashion using the UGT1A4cDNA (HUG-Br2). All cloned isoforms are undergoing analysis for substrate specificity.

Dr. Owens' group has shown that the UGT1A8cDNA-based protein, when expressed in the COS-1 cells, glucuronidated a new class of acceptor substrates. The rank in substrate turn over was flavones > anthroquinones > 17α-ethynlestradiol > complex alkyl phenols > biphenyls > bilirubin > benzo[a]pyrenes > α-naphthol. Flavones are known to inhibit growth of gastric and colon cancer cells.

Mutations in the Simple Phenol Transferase Isoform. Dr. Owens and her colleagues uncovered for the first time missense mutations in exons which code for phenol-selecting domains. They have uncovered two missense mutations in one allele of the *UGT1A6* gene of a human genome creating heterozygosity. The mutations, T181A and R184S, created *BsmI* and *BsoFI* endonuclease sites at the two respective mutations. The researchers verified heterozygosity by *BsmI*- and *BsoFI*-sensitivity of PCR-amplified genomic DNA from this individual. Upon screening some 98 individual DNA samples, they uncovered 33 individuals who were also heterozygous for the two mutations on one allele. One of these individuals also carried the R814S mutation on the second allele. Any manifestations of drug sensitivity or reactions by this individual could not be ascertained. The researchers predicted that 2/100 individuals are homozygous for the two linked mutations. They also carried out acceptor substrate activities with the UGT1A6 and the UGT1A6variant (A+ and S+) isoforms using 16 chemicals from 6 categories. They uncovered for the first time that both isozymes glucuronidated 1.1 to 6-fold better at pH 6.4 than at the typical pH 7.6 assay condition. The rank of chemical turnover for the UGT1A6 isoform was: simple phenols > coumarins > complex alkyl phenols > α-naphthol > acetaminophen. While the UGT1A6variant and UGT1A6 metabolized the simple phenols, coumarins and α-naphthol at similar rates; the variant enzyme metabolized the complex alkyl phenols and acetaminophen at 50% and 75% the normal rate, respectively. Size and bulkiness are factors which restrict glucuronidation by the isoforms; the pKa value of the hydroxyl group of all substrates is 7.8 and above. The UGT1A6 protein and its rat homologue were previously shown by other researchers to glucuronidate acetaminophen.

TATA Box Mutations in the Bilirubin Transferase Gene. A new class of mutations at the *UGT1* locus involving the TATA box transcriptional element of the *UGT1A1* gene was found by Dr. Owens' group to exist as heterozygous genotypes for both CN-I (CF) and CN-II (SM/TS) individuals; each patient contained a second missense mutation in the coding region. An allele contained either a TA deletion or TA insertion at the natural $[A(TA)_7A]$ TATA box element of both the CN-I and the CN-II patients. With the pCAT reporter gene fused with the A1 upstream regulatory region (-1.7 kb) containing either a wild-type $[A(TA)_7A]$, a TA deleted $[A(TA)_6A]$, or a TA inserted $[A(TA)_8A]$ at the TATA box, expression in HepG2 cells showed that the transcriptional rates were 65% and 20% normal, respectively, for the TA_6 and TA_8 mutants. In the coding region of these compound heterozygotes, the mutations I294T (SM), M310V(TS), R336W(CF), and I431T(TS) reduced glucuronidation rates to 22%, 40%, 3%, and 57% normal. From these *in vitro* results, it is predicted that the level of bilirubin transferase contributed by the two alleles of: CF (CN-I), SM (CN-II), and

TS (CN-II) is 11.5%, 21%, and 48% normal, respectively.

The evidence presented here by Dr. Owens'group on CN diseases and recently published data on Gilbert's individuals show that a single genetic defect (TATA box mutations) can exist among both of the CN diseases and the Gilbert's syndrome. Hence, the severity of the phenotypes depends upon the additive effect of at least two mutations whether homozygous or compound heterozygous. Dr. Owens believes that the inheritance, previously considered unclear, of all the bilirubin-related diseases can be accounted for by recessive inheritance of combinations of relative deleterious defects at the *UGT1A1* gene culminating in the phenotypes (CN-I, CN-II, and Gilbert's) observed in the population.

Structure-Function Relationship in the UGT1A1 Protein. Dr. Owens' group has shown with gel mobility analysis of radioactively labeled wild type or mutant TATA box oligos that a hepatocyte-specific nuclear protein(s) binds to $A(TA)_6A$ and $A(TA)_8A$ oligos at low protein concentration (0.02 to 0.5μg) but not to the wild type sequence. Higher levels of the protein(s) bound to $[A(TA)_8A]$ than to $[A(TA)_6A]$ to cause the band shift; this result paralleled the level of transcriptional inhibition by the two mutant TATA box elements. Furthermore, either element was displaced with excess nonradioactive TA_6 or TA_8 oligo in a concentration-dependent manner; the wild type sequences did not displace the mutant sequences. At higher protein levels (>1.0μg), a similar mobility shift was seen with wild type and the mutant oligos indicating that low nuclear protein level is required to demonstrate specific binding to the mutant oligos.

Dr. Owens' group continued to carry out structural analysis of the major bilirubin isoform UGT1A1 focusing on a highly hydrophobic region (microregion A) identified by a natural mutation uncovered in a CN-I patient. An in-frame phenylalanine deletion at codon 170 in exon 1 of *UGT1A1* disrupted a conserved di-phenylalanine among the bilirubin/bilirubin-like (UGT1A1-UGT1A5) isoforms. Using the RAO-ARGOS computer program designed to detect buried helices in membrane-anchored proteins the researchers discovered that the membrane-bound UGT1A1 protein contains a buried helix in microregion, a centered around the 170/171 di-phenylalanine. Prompted by the unique hydrophobic characteristics of this region in the UGT1A1-type proteins, they made select amino acid changes in this region and analyzed for the structure-function relationship. Although certain 170 substitutions satisfied the hydrophobicity requirement, Phe-170 was not replaceable. This hydrophobic region-- critical for bilirubin transferase activity-- reflects the most hydrophobic milieu in the substrate-selecting domain of the protein, presumably, for binding/interacting with the extremely hydrophobic-behaving bilirubin substrate.

Since all mammalian UDP-glucuronosyltransferases are endoplasmic reticulum-bound and, thus, difficult to purify for crystallography studies, a soluble surrogate protein would represent an alternative strategy for obtaining information on the active site of the UDP-glucuronosyltransferases. A bacuolviral UDP-glucosytransferase, lacking the 30 membrane-anchoring amino-acids at the carboxyl terminus, catalyzes a similar reaction; it conjugates glucose, donated by UDP-glucose, to ecdysone to inactivate the insect hormone. Conservation of a glycine-glycine di-peptide in all UDP-glucuronosyltransferase and in the baculoviral transferase and 61%/21% sequence similarity/identity within the equivalent common-end segment of the two types of proteins suggest that the baculoviral enzyme could serve as a protein model for tertiary structural studies. Dr. Owens' group has proceeded with generating ample amounts the UDP-glucosyltransferase for crystallization studies.

MOLECULAR MECHANISMS OF INFLAMMATION

The Section on Developmental Genetics under *Anil Mukherjee* conducts research into the molecular mechanisms of heritable diseases in which inflammation plays a major pathogenic role and into the development of novel strategies for their treatment. Thus, the investigations conducted in this Section are focused on the biochemistry, molecular biology and physiology of several important genes and their products which are related to the regulation of cellular functions which contribute to the process of inflammation. These genes are: (a) Uteroglobin (UG)/Clara cell 10kDa protein and its receptor (b) Phospholipases A_2 (PLA_2s) (c) group I & II PLA_2 receptors; and (d) Osteopontin (OPN) and cellular proto-oncogenes c-Src & c-fos as they relate to the pathogenesis of osteopetrosis.

Phospholipase A_2. For the past several years compelling evidence have been amassed to suggest that one of the major pathways for the initiation and propagation of inflammation involves the activation of PLA_2s. These enzymes also play an important regulatory role in many vital physiological processes, including cellular signal transduction, long-term potentiation and immunological activation. PLA_2s are key enzymes in the generation of arachidonic acid from cellular phospholipids and play a rate limiting role in the production of eicosanoids (eg. prostaglandins, leukotrienes, platelet activating factor and thromboxanes) which are well known mediators of inflammation. In addition, extracellular soluble PLA_2s have been found to have a cell surface receptor. Interaction of PLA_2 with its receptor appear to regulate several important cellular functions including smooth muscle contraction, important in the pathogenesis in inflammatory disease such as asthma. Thus, understanding the molecular mechanisms of regulation (i.e. activation and inhibition) of PLA_2s and its receptor is of great scientific and medical importance.

Regulation of Phospholipase A_2 Activity. Mukherjee and his associates have carried out pioneering studies on endogenous immunomodulatory proteins regulating the activity of PLA_2s. Some of these proteins are rabbit and human uteroglobin (hUG) also known as Clara cell 10kDa (cc10kDa) protein. Both of these proteins are not only important from the standpoint of their PLA_2-inhibitory, antiinflammatory effects, but also the genes coding for these proteins contain very strong promoters. These promoters are of considerable interest from the standpoint of standpoint of gene targeting, especially to the lung.

Osteopontin. This group has also initiated studies of an adhesive extracellular matrix protein, osteopontin (OPN). This regulation of OPN gene appears to be altered in a number of diseases including osteopetrosis, nephrolithiasis, and nearly all metastatic cancers. It has been reported that disruption of proto-oncogenes c-Src and c-fos results in osteopetrosis as a result of osteoclast dysfunction. Normal functioning of the osteoclast cells require osteopontin gene expression. Thus, Mukherjee and his collaborators studied OPN gene expression in c-Src and c-fos- disrupted (i.e. c-Src-/- and c-fos -/-) mice and found a dramatic lack of OPN gene expression. The biology and molecular mechanisms of certain forms of osteopetrosis are not clearly delineated and therefore effective therapies are not available. It is expected that ongoing investigations of this group will facilitate the development of new therapeutic approaches for this disease.

Targeted Disruption of Murine Uteroglobin Gene and Severe Renal Glomerular Disease. During the past year, Mukherjee and his associates have successfully disrupted the uteroglobin gene in mice by homologous recombination and obtained germline transmission of this null mutation. The $UG^{-}/_{-}$ mice, developing a severe inflammatory glomerular disease, become progressively cachectic and die within 4-5 weeks of birth. The pattern of transmission of this disease appears to be consistent with that of an autosomal dominant genetic disease. This disease in UG -/- mice bears a striking similarity to a recently reported hereditary glomerulopathy in huumans. At present Mukherjee and his associates are tring to delineate (i) the mechanism(s) of pathogenesis in the mouse model and determine (ii) whether UG gene mutation may underlie the pathogenesis of the hereditary human glomerulopathy.

Phospholipase A_2 Receptor. Last year, Mukherjee and his colleagues have successfully characterized a high-affinity group I PLA_2 receptor on several cell types, including the NIH 3T3 cells. This receptor appears to bind both porcine and *Naja naja* PLA_2s. Most significantly, it was found that binding of PLA_2 on NIH 3T3 cell surface dramatically increase their invasiveness of extracellular matrix (ECM) in an *in vitro* chemoinvasion assay. The mechanism of signal transduction via this receptor and its effect on cellular invasiveness are now being studied.

Subchromosomal Localization of the Human Uteroglobin Gene. This group localized the hUG gene on chromosome 11. During the past year they sought to determine the subchromosomal localization of this gene. It is now firmly established that hUG gene is localized in chromosome $11q^{12.3-13.1}$. This region of chromosome 11 harbors several important candidate genes including those of cervical carcinoma, atopy (allergic asthma), centrocytic lymphoma, Best's macular degeneration and possibly some tumor suppressors. Mukherjee and his associates cloned and characterized the hUG gene and are now investigating whether a mutation in this gene or in that of its receptor may be responsible for the pathogenesis of human diseases.

Transgenic Mice Expressing Antisense OPN. Since the physiological role of osteopontin is still obscure, Mukherjee and his colleagues, in a collaborative effort, generated OP-antisense transgenic mice in which the OP cDNA in an antisense orientation is placed under the regulation of MMTV-LTR promoter, inducible by dexamethasone. Preliminary results indicate that inhibition of OPN by its antisense mRNA expression results in severe abnormality in ductal differentiation in the mammary gland. As a result these animals do not produce milk. Since some expression of OPN may occur in antisense mice, Mukherjee and his associates have undertaken experiments to generate OPN knockout mice by homologous recombination. During the past year, they have successfully disrupted the OPN gene in murine ES cells. These cells will now be used to generate OP-null mice. These mice will be used to fully understand the physiological roles of OPN.

Protooncogenes and Osteopetrosis. In a separate study, using commercially mice in which proto-oncogene *c-fos* has been disrupted by homologous recombination, this group has demonstrated that manifestation of osteopetrotic phenotype by these mice, at least in part, may be associated with a dramatic reduction in OPN gene expression It has been suggested that osteopontin is essential for resorption of bone by osteoclasts. These cells secrete OPN which facilitates their attachment to bone surface. It is conceivable that lack of OPN gene expression by osteoclasts as this group has reported in c-Src-/- mice may impair their function in c-fos -/- mice as well. Ongoing *in vitro* studies are being carried out to delineate the relationship between *c-src* and *c-fos* proto-oncogenes to OPN gene expression in gene targe

Role of OPN in Human Coronary Restenosis. In is well established that atherosclerosis is the principal cause of heart attacks, stroke, gangrene and loss of function of extremities. It accounts for approximately 50% of all mortalities in the USA, Europe and Japan. Angioplasty procedures are increasingly used to reestablish blood flow in blocked atherosclerotic coronary arteries. A serious complication of these procedures is reocclusion (restenosis) which occurs in 30-50% of patients. Migration of coronary artery smooth muscle cells (CASMCs) to the site of injury caused by angioplasty and subsequent proliferation are suggested mechanisms of reocclusion. Using cultured human CASMCs and atherectomy tissues from patients, in a collaborative study, Mukherjee and his associates demonstrated that osteopontin (OPN) and one of its receptors ($\alpha_v\beta_3$ integrin) play critical roles in CASMC-migration, extracellular matrix-invasion, and proliferation. These effects are abolished when OPN or $\alpha_v\beta_3$ integrin gene-expression was inhibited by antisense s-oligonucleotide treatment of CASMCs. Similar results were also obtained when interaction between OPN and its receptor are blocked. These results establish an essential role for OPN and $\alpha_v\beta_3$ integrin in the pathogenesis of coronary reocclusion and raise the possibility that transient inhibition of OPN or $\alpha_v\beta_3$ integrin gene expression or blocking of OPN from its interaction with the receptor may have clinical application. Finally, invention reports have been filed on the UG-gene disrupted mice and a possible novel approach for preventing coronary restenosis.

A new group, the Unit on Molecular Dysmorphology, led by ***Forbes D. Porter***, was established this year. This group is interested in studying the molecular and biochemical processes that underlie dysmorphic syndromes. Initial work will focus on defining the role of two related LIM homeobox genes, Lhx2 and Lhx7, in development. Mice lacking Lhx2 have been generated by gene targeting in embryonic stem cells. Lhx2 mutant mice are anophthalmic, have forebrain anomalies, and die due to failure of definitive erythropoiesis. Using these mice, an effort is being made to clone genes that are regulated by Lhx2. By defining the genetic hierarchy in which Lhx2 functions,this groups hopes to gain insight into the molecular processes that underlie these malformations. Experiments will allow this group to determine how these two genes, independently and in combination, function during development.

In a line of investigation designed to complement the use of mouse models to study the molecular basis of dysmorphology, the Unit on Molecular Dysmorphology will be establishing a clinical protocol to evaluate children afflicted with Smith-Lemli-Opitz syndrome. These children have abnormal facial features, limb defects, genital anomalies, and brain malformations. Smith-Lemli-Opitz syndrome is thought to be due to a defect in cholesterol biosynthesis. By studying the biochemical and molecular perturbations in this syndrome, Dr. porter hopes to better understand the developmental defects it causes and to gain knowledge that can be applied clinically to help these patient and their families.

HERITABLE DISORDERS BRANCH CLINICAL PROTOCOLS

NUMBER	TITLE	PI	COMMENTS
76-CH-0238	Diagnosis and Treatment of Patients with Inborn Errors of Metabolism	Gahl	This is the screening protocol for the Heritable Disorders Branch. Allows Investigators to do preliminary evaluations which lead to additional clinical protocols.
78-CH-0093	Use of Cysteamine in the Treatment of Cysitinosis	Gahl	Initially provided the data for moving oral cysteamine to FDA approval. Currently patients are being seen every 2 years for long term follow-up.
92-CH-0034	Growth Hormone Therapy in Osteogenesis Imperfecta	Marini	Growth Hormone treatment has proved beneficial in some patients. Study is ongoing, but patient accrual is complete.
93-CH-0020	A Study of Collateral Disorders of Glycogen Storage Disease	Sidbury	This study evaluates unusual symptoms in some patients with Glycogen Storage Disease. Study is ongoing, but patient accrual is complete.
95-CH-0193	Clinical and Basic Investigations into Hermansky-Pudlak Syndrome	Gahl	The aims of this study focus on defining the myriad clinical features which characterize HPS, and developing effective treatment plans. Preliminary results documenting elevations in cholesterol and urinary dolichols are providing clues in the pursuit of the basic defect in HPS. Patient accrual is on-going.

HERITABLE DISORDERS BRANCH

Chief
Joan C. Marini, M.D., Ph.D.

Principal Investigators
Emma Jean deBrohun Butler, Ph.D.
Janice Y. Chou, Ph.D.
William A. Gahl, M.D, Ph.D.
Anil B. Mukherjee, M.D., Ph.D.
Ida S. Owens, Ph.D.

Tenure Track
Forbes D. Porter, M.D., Ph.D

Senior Fellows
Ohad Birk, M.D. (VA/CA-MSF)
Isabelle Cohen, Ph.D. (SF)
Jules A. Feledy, M.D. (CO/CA-MSF)
Ke-Jian Lei, Ph.D. (VS)
Mary L. McMaster, M.D. (CO/CA-MSF)
James M. Pash, Ph.D. (SSF)
Caroline C. Philpott, M.D. (CO/CA-MSF)
Vorasuk Shotelersuk, M.D. (VA/CA-MSF)

Post-Doctoral Fellows
Margaret Chamberlin, Ph.D. (IRTA)
David Chen, Ph.D. (IRTA)
Hungwen Chen, Ph.D. (VF)
Jeong Cho, Ph.D. (VF)
Marco Ciotti, M.D. (VF)
Paul Dawson, Ph.D. (VF)
Antonela Forlino, Ph.D. (VF)
Qi Hui Gong, Ph.D. (VS)
Gabriele Grassi, M.D. (VF)
Gopal C. Kundu, Ph.D. (IRTA)
Asim K. Mandal, Ph.D. (VF)
Geraldine McDowell, Ph.D. (SF)
Dibyendu Panda, Ph.D. (VF)
Qin Wang, M.D. (VF)
Ping Ye, M.D. (IRTA)
Chiun-Jye Yuan, Ph.D. (VF)
Zhongjian Zhang, M.D., Ph.D. (IRTA)
Chun Zhao, Ph.D. (NRC)

Pre-Doctoral Fellows
Kristin McAlister-Narten (Pre-IRTA)

Guest Researchers
Lawrence R. Charnas, M.D., Ph.D. (AS)
David Crane, M.D. (AS)
Ramasubbar Dhanireddy, M.D. (AS)
Lynn Duffy, M.D. (AS)
Anna Gajko, Ph.D. (AS)
Ozelm Goker-Alpan, M.D. (AS)
Juanru Guo, M.D. (AS)
Senator Hazelwood (HHMI)
Gordon Holt, Ph.D. (AS)
Takao Isogai, Ph.D. (AS)
Donna Krasnewich, M.D., Ph.D. (AS)
Sondra W. Levin, M.D. (AS)
Brian Mansfield, Ph.D. (AS)
Robert Pretzlaff, M.D. (AS)
Tsuneyuki Ubagai, Ph.D. (AS)

Scientist Emeritus
James B. Sidbury, M.D.

Senior Technical Specialists
Isa Bernardini
Chi-Jiunn Pan

HDB Annual Bibliography

Andersson HC, Kohn AD, Gahl WA, Kohn LD. Photoaffinity labeling of lysosomal membrane proteins with [^{125}I]-diiodotyrosine, a system h ligand. Biochem. Molec. Med. 1995; 55:71-73.

Byers PH, Marini JC, Gerber L. Frontiers in rehabilitation medicine: Osteogenesis Imperfecta, Overview of a Conference. Conn. Tissue Res. 1995; 31:253-255.

Chackalaparampil I, Peri A, Nemir M, McKee M, Lin PH, Mukherjee BB, Mukherjee AB. Cells *in vivo* and *in vitro* from osteopetrotic mice homozygous for *c-src* disruption show suppression of synthesis of osteopontin, a multifunctional extracellular matrix protein. Oncogene 1995; 12:1457-1467.

Chamberlin ME, Ubagai T, Mudd SH, Wilson WG, Leonard JV, Chou JY. Demyelination of the brain is associated with methionine adenosyltransferase I/III deficiency. J. Clin. Invest. Aug. 1996; 98(4):1021-1027.

Charnas LR, Marini JC. Neurologic profile in osteogenesis imperfecta. Conn. Tissue Res. 1995; 31:S23-S26.

Chen D, Guo J, Miki T, Tachibana M, Gahl WA. Molecular cloning of two novel *rab* genes from human melanocytes. Gene 1996; 174:129-134.

Chen H, Lin B, Chen CL, Johnson PF, Chou JY. Role of the Transcription Factor C/EBPβ in Expression of a Rat Pregnancy-specific Glycoprotein Gene. DNA Cell Biol. 1995; 14:681-688.

Chen HD, Chen H, Chou, JY, Gillis KD, Chen CL. Characterization of a prolactin-synthesizing rat pituitary cell line immortalized by the temperature-sensitive mutant SV40. Chin. J. Physiol. 1995; 4:201-209.

Chen F, Zhou J, Ritter JK, Bondy CA, Owens IS. Phenol and bilirubin UDP-glucuronosyltransferase RNAs are evenly distributed across the human liver lobule. Gastroenterology 1996; 111:472-480.

Ciotti M, Owens IS. Evidence for overlapping active sites for 17α-ethynlestradiol and bilirubin in the human major bilirubin UDP-glucuronosyltransferase. Biochemistry 1996; 35(31):10119-10124

Ciotti M, Obrary R, Martin MG, Owens IS. Genetic defects at the UGT1 locus associated with Crigler-Najjar Type I disease: Including a prenatal diagnosis. Amer J Med Genetics (In Press).

Earley K, Luo W, Qiu Y, Thompson NL, Chou J, Hixson DC, Lin SH. Identification of a new isoform of cell-cell adhesion molecule 105 (C-CAM), C-CAM4: a secretory protein with only one Ig domain. Biochem. J. 1996; 315:799-806.

Gahl WA. Nephropathic cystinosis. Pediatrics in Review 1996; (In press).

Gahl WA, Ingelfinger J, Mohan P, Bernardini I, Hyman PE, Tangerman A. Intravenous cysteamine therapy for nephropathic cystinosis. Pediatr. Res. 1995; 38:579-84.

Gahl WA, Potterf B, Durham-Pierre D, Brilliant MH, Hearing VJ. Melanosomal tyrosine transport in normal and pink-eyed dilution murine melanocytes. Pigment Cell Res. 1995; 8:229-233.

Gahl WA, Krasnewich DM, Williams JC. Sialidoses. In: Vinken PJ, Bruyn GA, eds. Handbook of Clinical Neurology. Amsterdam: Elsevier, 1996, chapter 25 (In press).

Gahl WA. Lysosomal Storage Diseases. In: Burg FD, Ingelfinger JR, Wald ER, Polin RA, eds. Current Pediatric Therapy. 15th ed. New York: WB Saunders Co., 1995; 376-7.

Grassi G, Marini JC. Ribozymes - Future tools for treatment of dominant diseases. Annals of Medicine. (in press)

Kaler SG, Das S, Levinson B, Goldstein DS, Holmes CS, Patronas NJ, Packman S, Gahl WA. Successful early copper therapy in Menkes disease associated with a transcript containing a small in-frame deletion. Biochem. Molec. Med. 1996; 57:37-46.

Kaler SG, Buist NRM, Holmes CS, Goldstein DS, Miller RC, Gahl WA. Early copper therapy in classical Menkes disease patients with a novel splicing mutation. Annals Neurol. 1995; 38:921-8.

Kimonis VE, Troendle J, Yang ML, Rose SR, Markello TC, Gahl WA. Effects of early cysteamine therapy on thyroid function and growth in nephropathic cystinosis. J. Clin. Endocrin. Metab. 1995; 80:3257-61.

Knight M, Ito Y, Chandrasekhar B, Takahashi K, Mukherjee AB. Purification of the human immunodeficiency virus-1 aspartyl protease substrate peptide.In: Amer. Chem. Soc. Symp. Series #593: Modern Countercurrent Chromatography. Conway, W.D. and Petroski, R.J. eds. Amer. Chem. Soc. Publ. 1995; Chapter 10: pp111-118.

Krasnewich DK, Holt GD, Brantly M, Skovby F, Redwine J, Gahl WA. Abnormal synthesis of dolichol-linked oligosaccharides in Carbohydrate-deficient Glycoprotein Syndrome. Glycobiology 1995; 5:503-510.

Kundu GC, Mukherjee AB. Evidence that porcine pancreatic phospholipase A_2 via its high-affinity receptor can stimulate extracellular matrix invasion by normal and cancer cells. J. Biol. Chem. (in press).

Kundu GC, Mantile G, Miele L, Cordella-Miele E, Mukherjee AB. Recombinant human uteroglobin suppresses cellular invasiveness via a novel class of high-affinity cell surface binding site. Proc. Natl. Acad. Sci. (USA) 1996; 93:2915-2919.

Lei KJ, Chen YT, Chen H, Wong LJC, Liu JL, McConkie-Rosell A, Van Hove JLK, Ou HC-Y, Yeh NJ, Pan LY, Chou JY. Genetic basis of glycogen storage disease type 1a: prevalent mutations at the glucose-6-phosphatase locus. Am. J. Hum. Gen. 1995; 57:766-771.

Lei KJ, Chen YT, Pan CJ, Ward JM, Mosinger B, Lee EJ, Westphal H, Chou JY. Glucose-6-phosphatase dependent substrate transport in the glycogen storage disease type 1a mouse. Nature Genetics 1996; 13:203-209.

Marini JC, Bordenick S, Chrousos GP. Endocrine aspects of growth deficiency in OI. Conn Tissue Res. 1995; 31:S55-S57.

Matern D, Niederhoff H, Brandis M, Chou JY. Verification of diagnosis in a 17-year old boy with clinical glycogen storage disease type 1a by mutational screening. J. Inherit. Met. Dis. 1996; 19:205-208.

Mathews JH, Pattabiraman N, Ward KB, Mantile G, Miele L, Mukherjee AB. Crystallization and characterization of the recombinant human Clara cell 10kDa protein. Proteins: Structure, Function and Genetics 1995; 20:191-196.

McDowell G, Town MM, van't Hoff W, Gahl WA. Genetic and physical mapping of the cystinosis gene. J. Mol. Med. 1996; (In press).

McDowell G, Isogai I, Tanigami A, Hazelwood S, Ledbetter D, Polymeropoulos MH, Lichter-Konecki U, Konecki D, Town MM, van't Hoff W, Weissenbach J, Gahl WA. Fine mapping of the cystinosis gene using an integrated genetic and physical map of a region within human chromosome band 17p13. Biochem. Molec. Med. 1996; 58:135-141.

Mukherjee AB, Miele L. Design of immunomodulatory peptides based on active site structures. In: Structure-based Drug Design (William Williams and David Weiner eds). CRC Press, Inc. 1995; pp.237-261

Mukherjee BB, Nemir M, Beninati S, Cordella-Miele E, Singh K, Chackaparampil I, Shanmugam V, DeVouge M, Mukherjee AB. Interaction of osteopontin with fibronectin and other extracellular matrix molecules. Ann. N.Y. Acad. Sci. 1995; 760:201-212.

Owens IS, Ritter JK, Yeatman MT, Chen F. The novel *UGT1* gene complex links bilirubin, xenobiotics, and therapeutic drug metabolism by encoding UDP-glucuronosyltransferase isozymes with a common carboxyl terminus. J Pharmocokinetics Biopharmaceutics (In press).

Panda D, Kundu GK, Lee BI, Peri A, Chackalaprampil I, Mukherjee BB, Li XD, Mukherjee DC, Seides S, Rosenberg J, Stark K, Mukherjee AB. Potential role of osteopontin and $\alpha_v\beta_3$ integrin in coronary restenosis. Nature (submitted).

Park C, Chamberlin ME, Pan C-J, Chou JY. Differential expression and butyrate response of human alkaline phosphatase genes are mediated by upstream DNA elements. Biochemistry, (in press).

Peri A, Dubin N, Dhanireddy R, Mukherjee AB. Uteroglobin gene expression in the rabbit uterus throughout gestation and in the fetal lung: Relationship between uteroglobin and eicosanoid levels in the developing fetal lung. J. Clin. Invest. 1995; 96:343-353.

Potterf SB, Muller J, Bernardini I, Tietze F, Kobayashi T, Hearing VJ, Gahl WA. Characterization of a melanosomal transport system in murine melanocytes mediating entry of the melanogenic substrate tyrosine. J. Biol. Chem. 1996; 271:4002-8.

Rutherfurd KJ, Chou JY, Mansfield BC. A motif in PSG11s mediates binding to a receptor on the surface of the promonocyte cell line THP-1. Mol. Endocrinol. 1995; 9:1297-1305.

Schneider JA, Clark KF, Greene AA, Reisch JS, Markello TC, Gahl WG, Thoene JG, Noonan PK, Berry KA. Recent advances in the treatment of cystinosis. J. Inher. Metab. Dis. 1995; 18:387-97.

Shanmugam V, Chackalaparampil I, Kundu GC, Mukherjee AB, Mukherjee BB. Sialic Acid Content Influences Specific Binding of Osteopontin to Temperature-sensitive Rat-1 (tsB77) Cell Surface. Biochemistry. (in press).

Shi ZZ, Habib GM, Rhead WJ, Gahl WA, He X, Sazer S, Lieberman MW. Mutations in the glutathione synthetase gene cause 5-oxoprolinuria. Nat. Genet. 1996; 14:361-365.

Su L, Mukherjee BB, Mukherjee AB. Expression of antisense osteopontin RNA inhibits tumor promoter-induced neoplastic transformation of mouse JB6 epidermal cells. Oncogene 1995; 10:2163-2169.

Theodoropoulos DS, Shawker TH, Heinrichs C, Gahl WA. Medullary nephrocalcinosis in nephropathic cystinosis. Pediatr. Nephrol. 1995; 9:412-8.

Ubagai T, Lei KJ, Huang S, Mudd SH, Levy HL, Chou JY. Molecular mechanisms of an inborn error of methionine pathway: methionine adenosyltransferase deficiency, J. Clin. Invest. 1995; 96:1943-1947.

Wang Q, Forlino A, Marini JC. Alternative splicing of COL1A1 mRNA leads to a partial null allele and two in-frame forms with structural defects in non-lethal Osteogenesis Imperfecta. J. Biol. Chem. Nov. 8, 1996; 271(45):28617-28623.

Wang Q, Marini JC. Antisense oligonucleotides selectively suppress expression of the mutant $\alpha 2$(I) collagen allele in OI type IV fibroblasts: A molecular approach to therapeutics of dominant negative disorders. J. Clin. Invest. 1996; 97:44-454.

Xing M, Miele L, Mukherjee AB. Arachidonic acid release from NIH 3T3 cells by group I phospholipase A_2: Involvement of a receptor-mediated mechanism. J. Cell. Physiol. 1995; 165:566-575.

Yergey AL, Vieira NE, Abrams SA, Marini JC, Goans RE. Use of stable isotopic tracers in studies of whole body calcium metabolism. Conn. Tiss. Res. 1995; 31:291-293.

Zhang Z, Ward JM, Lee E, Kundu GC, Yuan P, De Mayo F, Westphal H, Mukherjee AB. Mice homozygous for uteroglobin gene disruption (UG -/-) develop severe renal glomerular disease. (to be submitted).

Zhang Z, Zimonjic DB, Popescu NC, Wang N, Gerhard D, Stone E, Arbour NC, de Vries MA, Scheffer H, Garritsen J, Collie' J, ten Kate LP, Mukherjee AB. Human uterogobin gene: Its structure, subchromosomal localization, polymorphism and possible role in disease processes. DNA & Cell Biol. (in press).

Project Numbers - Annual Report 1996

HERITABLE DISORDERS BRANCH (HDB)

ZO1 HD 00131-22	Human Biochemical Genetics William A. Gahl, M.D., Ph.D.
Z01 HD 00137-22	Regulation and Expression of the UDP-Glucuronosyltransferase Gene Family Ida S. Owens, Ph.D.
ZO1 HD 00404-14	Cell and Sulfur Metabolism in Fibroblasts of Genetic Diseases Jean DeBrohun Butler, Ph.D.
ZO1 HD 00408-13	Heritable Disorders of Connective Tissue Joan C. Marini, M.D., Ph.D.
ZO1 HD 00910-17	Study of Uteroglobin, Phospholipase A_2, and Osteopontin Genes Anil B. Mukherjee, M.D., Ph.D.
ZO1 HD 00912-17	Gene Regulation and Cellular Differentiation Janice Y. Chou, Ph.D.

ZO1 HD 00139-45
then use **00418-499**

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00131-22 HDB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Human Biochemical Genetics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: William A. Gahl, M.D., Ph.D., Head, Section on Human Biochemical Genetics, HDB, NICHD
Others: I. Bernardini, Chemist, HDB, NICHD
D. Chen, Ph.D., IRTA Fellow, HDB, NICHD
T. Isogai, Ph.D., Special Volunteer, HDB, NICHD
G. McDowell, Ph.D., IRTA Fellow, HDB, NICHD
P. Ye, Ph.D., IRTA Fellow, HDB, NICHD
R. Pretzlaff, M.D., Special Volunteer, HDB, NICHD
S. Hazelwood, Special Volunteer, HDB, NICHD
V. Shotelersuk, M.D., Medical Staff Fellow, HDB, NICHD

COOPERATING UNITS *(if any)*

See Attached

LAB/BRANCH

Heritable Disorders Branch

SECTION

Section on Human Biochemical Genetics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Members of the Section, having mapped the cystinosis gene to chromosome 17p, have now narrowed the region of the gene to a 130 kb span by identifying new polymorphic markers and informative recombinants. Several nonpolymorphic markers have been mapped to the area by radiation hybrid analysis. Two P1 clones span the cystinosis gene region, flanked by markers D17S1828 and D17S2167. Since they overlap, the cystinosis gene must be located on one of the clones. The identification and testing of candidate genes continues.
2. We have identified 24 rab proteins (small, monomeric GTP-binding proteins) in melanocytes. Rab30 represents a novel protein of 203 amino acids, and rab22b appears to be an isoform of the human homologue of canine rab22. Furthermore, rab27a and rab27b, which comprise a rab subfamily expressed in both melanocytes and platelets, are candidates for involvement in human and mouse disorders of pigment dilution and a platelet storage pool defect.
3. A tyrosine transport system has been characterized in the melanosomes of murine melanocytes. It exhibits countertransport and saturability, with an apparent Km for tyrosine of 54 μM. In its substrate preferences, it resembles lysosomal system h of rat thyroid FRTL-5 cells. The melanocyte's melanosomal transport system appears critical for the production of the pigment melanin.
4. The Section has examined over 30 patients with Hermansky-Pudlak syndrome, a disorder consisting of albinism, a bleeding diathesis, and lysosomal storage of ceroid lipofuscin. Half the patients have been Puerto Rican. Dolichols have been identified as components of the ceroid lipofuscin and several patients have exhibited increased levels of cholesterol and coenzyme Q10, all suggesting involvemnt of the cholesterol synthetic pathway in this disorder. Clinical characterization of the disease may point to avenues of therapy.

Cooperating Units:

F. Tietze, Laboratory of Molecular and Cellular Biology, NIDDK
J. Schneider, University of California at San Diego
J. Thoene, University of Michigan
M. Kaiser-Kupfer, Clinical Branch, NEI
L. Charnas, Neurological Associates, Grand Junction, Colorado
D. Goldstein, Clinical Neuroscience Branch, NINDS
A. Mohan, George Washington University Medical Center
W. van't Hoff, Guy's Hospital, London
M. Brantly, NHLBI
M. Grompe, Oregon Center for Health Sciences, Portland
M. Polymeropoulos, NCHGR
D. Ledbetter, NCHGR
A. Tangerman, St. Radboud University Hospital, Nijmegen, Netherlands
C. Waechter, University of Kentucky College of Medicine, Lexington, KY
R. Miller, Bethesda Naval Hospital
T. Markello, Medical College of Virginia, Richmond, VA
V. Hearing, National Cancer Institute
R. Boissy, University of Cincinnati
D. Theodoropoulos, Interinstitute Genetics Program, NIH Clinical Center
F. Skovby, Rigshospitalet, Copenhagen, Denmark
W. Rhead, University of Iowa
N. Buist, University of Oregon
H. Fales, NHLBI
G. Holt, Oxford Glycosystems, Gaithersburg, MD
R. Seppala, University of Oulu, Finland
D. Krasnewich, NIMH
T. Shawker, Dept. of Radiology, NIH Clinical Center
J. Ingelfinger, Massachusetts General Hospital, Boston, MA
M. Brilliant, Fox Chase Cancer Research Center, Philadelphia, PA
J. Williams, Children's Hospital of Los Angeles, Los Angeles, CA
M. Lieberman, Baylor College of Medicine, Houston, TX
T. Miki, NCI
M. Tachibana, NIDCD
D. Konecki, Marshfield Center for Medical Genetics, Marshfield, WI
U. Konecki, Marshfield Center for Medical Genetics, Marshfield, WI
S. Chandrasekharappa, NCHGR
E. Green, NCHGR
J. Zimmerberg, NICHD
H. Andersson, Tulane University Medical Center, New Orleans, LA

Project Description

Objectives:

The goals of this project are to: 1) isolate the cystinosis gene and perform mutational analysis of the gene in affected individuals; 2) provide diagnosis and therapy for the renal and nonrenal complications of nephropathic cystinosis; 3) characterize new and rare metabolic disorders in man, such as Carbohydrate-Deficient Glycoprotein Syndrome, by describing patients clinically and determining the basic defect involved; 4) describe defects of lysosomal membrane transport and other new lysosomal storage diseases based on characterization of the stored material; 5) develop new methodologies for the analysis of carbohydrates in the urine of patients with rare disorders of carbohydrate metabolism; 6) determine the characteristics of tyrosine transport across normal murine melanosomal membranes; 7) identify the rab proteins expressed in cultured human melanocytes; 8) identify the stored material known as ceroid lipofuscin in Hermansky-Pudlak syndrome; 9) clinically and biochemically characterize Puerto Rican and non-Puerto Rican patients with Hermansky-Pudlak syndrome.

Methods Employed:

The principal methods employed are: 1) spectrophotometric, radiochemical, and fluorimetric enzyme assays; 2) cell culture techniques; 3) differential centrifugation; 4) quantitative ion exchange chromatography of amino acids in physiologic fluids; 5) radioisotopic measurements; 6) light, phase contrast, and electron microscopy; 7) column, thin layer, affinity chromatography of lipids and/or carbohydrates; 8) isoelectric focusing and polyacrylamide gel electrophoresis; 9) mass spectroscopy of unknown compounds; 10) pulsed amperometric (Dionex) detection of carbohydrates; 11) high performance liquid chromatography of dolichols and urinary oligosaccharides; 12) cloning and sequencing using plasmids, cDNA library screening, and expression systems; 13) reverse-transcriptase PCR and direct PCR; 14) mutation analysis using southern and northern blotting and single-stranded conformational polymorphism (SSCP) analysis; 15) linkage analysis using polymorphisms of microsatellite repeats; 16) genomic DNA and expression library screening; 17) construction of yeast artificial chromosome contigs; 18) pulsed field gel electrophoresis; 19) radiation hybrid mapping of microsatellite markers; 20) exon trapping; 21) shotgun sequencing of P1 clones; 22) searching data banks for sequence homology with candidate genes.

Major Findings:

Cystinosis.

Nephropathic cystinosis is an autosomal recessive disorder due to defective transport of the amino acid cystine out of lysosomes. Cystinosis causes renal failure at approximately 10 years of age, but the cystine-depleting agent cysteamine retards or prevents this deterioration and enhances growth. Without cysteamine therapy, a variety of nonrenal complications plague post-transplant patients after their second decade of life.

Dr. Geraldine McDowell continues to pursue the gene for cystinosis. Over the past 3 years, she has spearheaded an international collaborative effort which made use of linkage analysis to map the cystinosis gene to a 3.1 cM region of chromosome 17p13 flanked by the microsatellite markers D17S1828 and D17S1798. In collaboration with Drs. David Ledbetter and Akira Tanigami of the NCHGR, Dr. McDowell created a yeast artificial chromosome (YAC) contig between these markers and reported an integrated genetic and physical map of the region. Using one pertinent YAC clone, 898A10, Dr. McDowell and Dr. Takao Isogai identified new polymorphic microsatellite repeat markers in the cystinosis gene region. One such marker, D17S2167, was localized by radiation hybrid analysis to within 10.2 cR_{8000} of D17S1828. (Radiation hybrid mapping involves the use of a panel of rodent/human hybrids in which the human DNA has been cleaved by x-ray exposure. Markers close to each other are less likely to have a break between them and, therefore, will be lost or retained together upon fusion with rodent cells. Distant markers are more likely to have a break between them and will be lost or retained independently. The presence of pertinent markers in the cystinosis gene area on the 83 hybrids of the Stanford G3 radiation hybrid panel was determined by PCR assay. The order and relative distances of these markers were determined by multipoint analysis using the program RHMAX4K.) Haplotype analysis in two separate informative families revealed recombination events which placed the cystinosis gene between markers D17S1828 and D17S2167.

Next, three P1 clones, each containing approximately 70 kb of human DNA, were isolated and shown to contain either D17S1828 or D17S2167. Two of these P1 clones overlapped, since they shared nonpolymorphic markers and yielded some identically sized fragments on digestion with restriction endonucleases *EcoR1* and *HindIII*. Therefore, the 2 P1 clones comprise a contig across the region of interest, i.e., one of the P1 clones must contain the cystinosis gene. In addition, the area of interest should be approximately 130 kb in size; this was verified by pulsed-field gel electrophoresis of the P1 clones. The P1 DNA is now being used in a variety of gene-seeking strategies, including screening a cDNA library, probing northern blots containing RNA from several cystinosis patients, 3'-exon trapping, and partial or complete sequencing followed by examination of computer data banks for anonymous expressed sequence tags. The ultimate cloning of the cystinosis gene should reveal how a functional lysosomal transport protein is synthesized, targetted, processed, and integrated into the lysosomal membrane.

Clinical investigators in the Section continue to provide care for over 100 patients with nephropathic cystinosis who are admitted to the NIH Clinical Center every two years. This patient population will reveal how long children treated with cysteamine can maintain renal function without requiring an allograft procedure. Post-transplant patients will demonstrate to us whether chronic cysteamine therapy prevents or delays the nonrenal complications of the disease, which include retinal blindness, a distal vacuolar myopathy, swallowing difficulty, and pancreatic insufficiency. In collaboration with Dr. Muriel Kaiser-Kupfer of the National Eye Institute, members of the Section continue to assist in the provision of cysteamine eyedrops, which dissolve the cystine crystals in patients' corneas. The NIH remains the only source of these eyedrops for over 100 patients. Both Dr. Gahl and Dr. Kaiser-Kupfer are cooperating with a pharmaceutical company in bringing this medicine to New Drug Approval by the Food and Drug Administration; this would mean that cysteamine eyedrops could be prescribed throughout the country. At the same time, Dr. Gahl is assisting in the approval process for cysteamine in the European Community, whose FDA equivalent is now considering an application submitted by Orphan Europe.

Disorders of Carbohydrate Metabolism.
Screening of urine specimens for possible carbohydrate storage disorders such as oligosaccharidoses and mucopolysaccharidoses remains problematic. Drs. Geraldine McDowell and Gordon Holt previously developed a method of desalting urine on mixed-bed resins followed by trifluoracetic acid hydrolysis and analysis by HPLC. They have expanded upon this technique by tagging the resulting monosaccharides with 2-aminobenzoic acid (2AA). 2AA-labeled monosaccharides are separated by conventional reversed phase HPLC (C18) under isocratic conditions and quantitated on line with a standard fluorescence detector. All neutral monosaccharide components of hydrolyzed human urine are detected with very high sensitivity (<50 pmol) by this method. Monosaccharide components of glycosaminoglycans (e.g., neutral sugars and uronic acid with or without sulfate) are resolved under the same conditions. Thus, this method can be employed to easily screen for both oligosaccharidoses

and mucopolysaccharidoses using instrumentation available in most clinical diagnostic laboratories. The technology is currently being tested using urines from patients with known disorders of carbohydrate storage.

The same method, along with thin layer chormatography and pulsed amperometric detection (Dionex), is being applied to the study of patients with carbohydrate storage disorders of unknown etiology. Typical patients include those with geleophysic dysplasia, Schimke immuno-osseous dysplasia, increased storage of mucin type oligosaccharides, and a variety of other enigmatic defects. Both fibroblasts and urine are being analyzed in this respect.

The Section remains the world's resource center for the diagnosis of new and established disorders of sialic acid metabolism. Samples from patients with a wide range of clinical presentations are analyzed by R. Seppala, and members of the Section have written the definitive chapter on sialidoses for the most authoritative reference text of neurologists, the Handbook of Clinical Neurology.

Rab proteins.

Rab proteins represent a family of nontransforming monomeric GTP-binding proteins in the ras superfamily. Similar to ras, these proteins have four highly conserved regions (designated I to IV) involved in GTP binding and hydrolysis. They also contain a consensus carboxy-terminal amino acid sequence containing two cysteine residues. Geranyl-geranylation of a carboxy-terminal cysteine allows for anchoring of the rab to the plasma membrane or to a vesicular membrane. Subsequently, in a fashion not fully elucidated, rabs regulate the critical intracellular processes of membrane trafficking and vesicular fusion and targeting. The expression of certain rab genes may prove critical to the differentiated function of cells requiring unique vesicle targeting or fusion pathways.

Melanocytes typify such differentiated cells. Their unique vesicles, melanosomes, perform the cells' primary function, i.e., they produce melanin pigment. However, the rab proteins of melanocytes have not been catalogued. To accomplish this, Dr. David Chen used a PCR-based strategy to isolate partial rab cDNA sequences from pigmented human melanoma cells. He used degenerate PCR primers designed based on the amino acid sequences WDTAQ and GNKXD, two GTP-binding domains that are strictly conserved in all members of the human rab GTPases. The distances between these two domains are also nearly invariable.

Dr. Chen identified 24 different small GTP-binding proteins in two rounds of screening; eight of these accounted for approximately 85% of the GTP-binding protein expression of the melanoma cells. These eight were rap1A, rab11, rab1A, ras-like protein, R-ras, rab13, rho, and LLrep3. In addition, rab27b, which shows strong homology to rat ram p25 (see below), was quite abundant.

Two other rab genes were isolated and given names designating their novel status. One, rab30, codes for a protein of 203 amino acids with minimal homology to previously documented GTPases. Another, rab22b, appears to be an isoform of the human homologue of canine rab22. The complete cDNA sequences of these genes were determined by a combination of 3'-RACE (rapid amplification of cDNA ends), 5'-RACE, and cDNA library screening. Both of the mRNAs of rab30 and rab22b displayed a nearly ubiquitous pattern of expression across the range of tissues examined. Rab22b and rab30 were mapped to chromosomes 18 and 11, respectively.

In addition, the complete cDNAs of two rab isoforms, rab27a and rab27b, were isolated from human melanoma cells and melanocytes. Rab27a is the human homolog of a rat megakaryocyte rab called ram p25. Rab27b corresponds to a small GTP-binding protein, c25KG, which was previously purified from platelets but whose cDNA had not been cloned. Sequence comparisons with known rabs indicate that rab27a and rab27b comprise a melanocyte/platelet subfamily within the rab family.

In addition, rab27a was expressed in a large variety of cell and tissue types, excluding brain, while rab27b manifested itself primarily in testis. Dr. Chen expressed and purified rab27a and rab27b in bacteria and demonstrated their GTP-binding activities; these proteins can now be used for antibody production and studies of the substrate specificities with respect to geranylgeranyl transferase.

The expression of rab27a and rab27b in both melanocytes and platelets makes them candidates for involvement in mouse and human mutations causing the combination of pigment dilution and a platelet storage pool defect. Murine examples include *pale ear*, which corresponds both phenotypically and genetically to the human disorder, Hermansky-Pudlak syndrome (HPS). HPS consists of the triad of tyrosinase-positive albinism, storage pool deficiency due to absent platelet dense bodies, and accumulation of ceroid lipofuscin in cellular lysosomes. Each of these abnormalities could be related to a vesicular membrane defect. Another mouse mutant, *beige*, serves as a model for the human disorder Chediak-Higashi disease (CHD), in which partial albinism results from giant melanosomes, platelet dense bodies are reduced in number, and frequent pyogenic infections occur due to giant, fused, dysfunctional lysosomes. Microscopic and histochemical observations of various CHD cell types have suggested that the CHD and *beige* mutations represent an abnormality in fusion or fission of late endocytic structures. Hence, proteins such as the rabs which affect membrane fusion and targeting are candidates for the gene products of CHD or *beige*. Moreover, because rab27a and rab27b are expressed in platelets as well as melanocytes, they, in particular, should be considered in these disorders. In collaboration with Dr. Ray Boissy of the Medical College of Cincinnati, the expression of rab27a and rab27b in HPS and CHD cultured melanocytes is being examined by northern blot analysis and by RT-PCR.

Melanosomal Tyrosine Transport.

The Section maintains an interest in melanosomal tyrosine transport for various reasons. The movement of small molecules across the melanosomal membrane should resemble transport across the lysosomal membrane, which has been a longstanding focus of the group. In addition, impaired melanosomal transport of one molecule, tyrosine, may cause a certain form of albinism, since tyrosine is a requisite precursor of the pigment melanin. Therefore, in collaboration with Dr. Vincent Hearing and Brian Potterf, pigmentologists in the National Cancer Institute, members of the Section have characterized melanosomal tyrosine transport in cultured mouse melanocytes. In critical experiments, melanosomes were preloaded with tyrosine (using nonradioactive tyrosine methylester) to demonstrate countertransport of 10 μM [^{3}H]tyrosine, indicating carrier-mediated transport. In other experiments, melanosomal tyrosine uptake was shown to be saturable, with an apparent K_m for tyrosine transport of 54 μM and a maximal velocity of 15 pmol tyrosine/unit hexosaminidase/min. (Hexosaminidase serves as a marker for the number of melanosomes present.) Transport was temperature-dependent (E_a= 7.5 kcal/mol) and showed stereospecificity for the L-isomer of tyrosine. Aromatic, neutral hydrophobic compounds (such as tryptophan and phenylalanine), as well as small, bulky neutral amino acids (such as leucine, isoleucine, and methionine) competed for tyrosine transport. Tyrosine transport was inhibited by the classical system L analogue, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid and by monoiodotyrosine, but not by cystine, lysine, glutamic acid, or 2-(methylamino)-isobutyric acid. Tyrosine transport showed no dependence on Na^+ or K^+, and did not require an acidic environment or the availability of free thiols. These results demonstrate the existence of a neutral amino acid carrier in murine melanocyte melanosomes which resembles the rat thyroid FRTL-5 lysosomal system *h*. This transport system appears critical to the production of melanin, and a defect in the melanosomal tyrosine carrier could result in some type of albinism.

Hermansky-Pudlak Syndrome.

Hermansky-Pudlak syndrome (HPS) consists of the triad of albinism, a bleeding diathesis, and lysosomal storage of ceroid lipofuscin. The albinism is tyrosinase-positive and of extremely variable severity. The bleeding diathesis is due to a platelet storage pool defect. Specifically, the platelet dense bodies, which contain ADP, ATP, calcium, and serotonin, are virtually absent in HPS. These intracellular vesicles are responsible for the secondary aggregation response of platelets. Ceroid lipofuscin is a poorly-defined, lipid-protein substance stored inside lysosomes which fluoresces in a characteristic fashion. It appears to accumulate progressively in HPS. The basic defect remains unknown, but probably involves the membranes of the melanosome, dense body, and lysosome, all of which are impaired in HPS. This autosomal recessive disease is most common in Puerto Rico, where the incidence in some areas is 1 in 1800 live births. Clinical manifestations are extremely variable, but the most serious and insidious medical complication is pulmonary fibrosis, presumably due to the inexorable accumulation of ceroid lipofuscin. This generally proves fatal in the fifth decade of life. Another organ affected by the storage is the intestine, which manifests a granulomatous colitis in some patients. Diagnosis of HPS rests upon finding absent or rare platelet dense bodies in an albino patient with a storage pool defect.

In August of 1995, a clinical protocol was established by the Section to investigate HPS, with the goals of determining the basic defect, better characterizing the disorder among Puerto Rican and non-Puerto Rican patients, and developing therapy. Over 30 patients age 3 to 60 years of age, half of Puerto Rican ancestry, have been studied at the NIH Clinical Center. Insights into the basic defect have followed the lead that renal tubular epithelial cells sloughed into the urine of HPS patients contain dolichols, hydrophobic lipids composed of chains of isoprene units. Dolichols are offshoots of the cholesterol synthetic pathway, and several children with HPS have displayed high cholesterol levels. Many patients also exhibit elevated blood concentrations of coenzyme Q_{10}, another product of the cholesterol synthetic pathway. Finally, a collaboration with Dr. Josh Zimmerberg's group has revealed an increased fusability of HPS red blood cell membranes, consistent with an increased content of highly hydrophobic lipids such as dolichols. All this circumstantial evidence prompts further pursuit of the hypothesis that the basic defect in HPS involves up-regulation of the cholesterol synthetic pathway.

The clinical characterization of HPS includes extensive blood and urine chemistries as well as specialty consultations. As a group, the HPS patients have exhibited normal renal, hepatic, and endocrine function. As expected, platelet aggregation studies have been abnormal, but lymphocyte phenotyping results appear within the normal range. Visual acuity ranges from 20/100 to 20/400, with variable degrees of nystagmus and iris transillumination. Granulomatous colitis has been a prominent feature in only 10% of patients. Dermatologic examination has revealed extremely variable degrees of pigmentation, increased hirsutism, and prominent nevi in several patients. Pulmonary function tests have been abnormal in individuals as young as 12 years old, and show a restrictive pattern in the majority of patients over 30 years of age. Some patients have characteristically thickened bronchial septa on high-resolution CT scans of the lung. Pulmonary lavage performed on 5 adults with HPS has shown a 5-10 fold increase over normal in the number of cells recovered. Further analysis of the inflammatory mediators produced by these cells could indicate the type of therapy to which HPS patients will respond.

Significance to Biomedical Research and the Program of the Institute:

This Section's investigations, directed toward the etiology, diagnosis, and treatment of inborn errors of metabolism, fulfill important objectives of biomedical research in general and address primary concerns of the Institute. The Section serves as a widely recognized repository for clinical and biochemical expertise in the field of cystinosis, for biochemical analysis of disorders of sialic acid metabolism, and for methodology in the fields of carbohydrate analysis and lysosomal membrane transport. Regarding cystinosis, the Section follows over one-third of all the known patients in the United States and serves as the sole source of cysteamine eyedrops (through the National Eye

Institute) for over 100 patients. The Section's studies on the cloning of the lysosomal membrane's cystine transporter will permit the different mutations in cystinosis to be defined at the molecular level, and will help determine how carriers targeted for the lysosomal membrane reach their final destination.

The Section's investigations into disorders such as Hermansky-Pudlak Syndrome may reveal new pathways in human cell biology and allow for new methods of treatment for this unique human condition. As for all rare diseases, clinical investigations provide the only hope of advancing therapy, and also offer the potential for deeper understanding of the biochemical and cellular mechanisms involved. These comprise important aspects of the Institute's mission.

Proposed Course:

Future efforts will include: 1) Attempts to isolate the cystinosis gene, known to reside on one of 2 P1 clones, by exon trapping, shotgun sequencing of the P1 clones, and screening of cDNA libraries using markers in the region of interest; candidate genes will be tested by northern blot and RT-PCR analysis; 2) Mutational analysis of cystinosis patients once the gene is cloned; 3) Continuation of clinical followup of cystinosis patients every 2 years; 4) Use of newly-developed methodologies in urinary carbohydrate analysis to define the basic defects in a variety of carbohydrate disorders of unknown etiology; 5) Determination of the pattern of expression of rab genes in human melanocytes cultured from normal, HPS, and CHD patients; 6) Investigation into the basic defect in Hermansky-Pudlak syndrome, specifically the cause of ceroid lipofuscin storage, using fibroblasts and melanocytes of affected patients to study the cholesterol synthetic pathway; 7) Development of treatment regimens for the pulmonary disease of HPS.

Clinical Trials:

Protocols of the Section on Human Biochemical Genetics currently approved by the NICHD Institutional Review Board include:

78-CH-93 - "Use of Cysteamine in the Treatment of Cystinosis"

76-CH-238 - "Diagnosis and Treatment of Patients with Inborn Errors of Metabolism"

95-CH-193 - "Clinical and Basic Investigations into Hermansky-Pudlak Syndrome"

94-CH-201- "Glutathione Monoethyl Ester in the Therapy of Human Glutathione Synthase Deficiency"
Terminated; no patients in past year.

90-CH-149 - "Copper Histidinate Therapy in Menkes' Disease"
Transferred to NINDS.

William A. Gahl, MD, PhD, is the principal investigator for the first four protocols. In addition, Dr. Gahl is associate investigator for National Eye Institute project 86-EI-62, "A Double-Masked Controlled Randomized Clinical Trial of Topical Cysteamine in the Treatment of Corneal Cystine Crystal Accumulation in Cystinosis".

Publications:

Andersson HC, Kohn AD, Gahl WA, Kohn LD. Photoaffinity labeling of lysosomal membrane proteins with [^{125}I]-diiodotyrosine, a system h ligand. Biochem Molec Med 1995; 55:71-3.

Chen D, Guo J, Miki T, Tachibana M, Gahl WA. Molecular cloning of two novel *rab* genes from human melanocytes. Gene 1996 (In press).

Gahl WA. Lysosomal Storage Diseases. In: Burg FD, Ingelfinger JR, Wald ER, Polin RA, eds. Current Pediatric Therapy. 15th ed. New York: WB Saunders Co., 1995, 376-7.

Gahl WA. Nephropathic cystinosis. Pediatrics in Review 1996 (In press).

Gahl WA, Ingelfinger J, Mohan P, Bernardini I, Hyman PE, Tangerman A. Intravenous cysteamine therapy for nephropathic cystinosis. Pediatr Res 1995; 38:579-84.

Gahl WA, Krasnewich DM, Williams JC. Sialidoses. In: Vinken PJ, Bruyn GA, eds. Handbook of Clinical Neurology. Amsterdam: Elsevier, 1996, chapter 25 (In press).

Gahl WA, Potterf B, Durham-Pierre D, Brilliant MH, Hearing VJ. Melanosomal tyrosine transport in normal and pink-eyed dilution murine melanocytes. Pigment Cell Res 1995; 8:229-33.

Kaler SG, Buist NRM, Holmes CS, Goldstein DS, Miller RC, Gahl WA. Early copper therapy in classical Menkes disease patients with a novel splicing mutation. Annals Neurol 1995; 38:921-8.

Kaler SG, Das S, Levinson B, Goldstein DS, Holmes CS, Patronas NJ, Packman S, Gahl WA. Successful early copper therapy in Menkes disease associated with a transcript containing a small in-frame deletion. Biochem Molec Med 1996; 57:37-46.

Kimonis VE, Troendle J, Yang ML, Rose SR, Markello TC, Gahl WA. Effects of early cysteamine therapy on thyroid function and growth in nephropathic cystinosis. J Clin Endocrin Metab 1995; 80:3257-61.

Krasnewich DK, Holt GD, Brantly M, Skovby F, Redwine J, Gahl WA. Abnormal synthesis of dolichol-linked oligosaccharides in Carbohydrate-deficient Glycoprotein Syndrome. Glycobiology 1995; 5:503-10.

McDowell G, Isogai I, Tanigami A, Hazelwood S, Ledbetter D, Polymeropoulos MH, Lichter-Konecki U, Konecki D, Town MM, van't Hoff W, Weissenbach J, Gahl WA. Fine mapping of the cystinosis gene using an integrated genetic and physical map of a region within human chromosome band 17p13. Biochem Molec Med 1996 (In press).

McDowell G, Town MM, van't Hoff W, Gahl WA. Genetic and physical mapping of the cystinosis gene. J Mol Med 1996 (In press).

Potterf SB, Muller J, Bernardini I, Tietze F, Kobayashi T, Hearing VJ, Gahl WA. Characterization of a melanosomal transport system in murine melanocytes mediating entry of the melanogenic substrate tyrosine. J Biol Chem 1996; 271:4002-8.

Schneider JA, Clark KF, Greene AA, Reisch JS, Markello TC, Gahl WG, Thoene JG, Noonan PK, Berry KA. Recent advances in the treatment of cystinosis. J Inher Metab Dis 1995; 18:387-97.

Shi Z-Z, Habib GM, Rhead WJ, Gahl WA, He X, Sazer S, Lieberman MW. Mutations in the glutathione synthetase gene cause 5-oxoprolinuria. Nat Genet 1996 (In press).

Theodoropoulos DS, Shawker TH, Heinrichs C, Gahl WA. Medullary nephrocalcinosis in nephropathic cystinosis. Pediatr Nephrol 1995; 9:412-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00137-22 HDB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Organization, Regulation, and Expression of the UDP-Glucuronosyltransferase Gene Family

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ida S. Owens	Head	HDB, NICHD
Others:	Marco Ciotti	Visiting Fellow	HDB, NICHD
	Jeong Cho	Visiting Fellow	HDB, NICHD
	Qi-Hui Gong	Technician	HDB, NICHD

COOPERATING UNITS *(if any)*

Imperial College of Science, Technology, and Medicine, London, England (Dr. D.R. O'Reilly)
University of Rochester, Department of Pharmacology, (Dr. J. P. Jones).

LAB/BRANCH

Heritable Disorders Branch

SECTION

Section on Genetic Disorders of Drug Metabolism

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	3.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our description of the *UGT1* locus is now essentially complete. Its description has been critical to determining defects in patients with Crigler-Najjar(CN) diseases. The original version of the gene complex, UGT1A-UGT1F and recently renamed UGT1A1-UGT1A6, codes for bilirubin, bilirubin-like, and phenolUDPglucuronosyltransferases. In the 5' region of the locus, six different exons 1, each with an upstream promoter and each encoding the amino terminus of an isoform, are arrayed in series with 4 common exons encoding six identical carboxyl termini in the 3' region. We have extended the locus by identifying, sequencing, and mapping six additional exons 1 coding for UGT1A7-UGT1A12P genes. The newly identified exons are 86-91% identical to each other and distantly related, 50-53% identical, to the first six exons 1. The TATA box element upstream of the final six exons 1 is either AAT_9AAAT or $AT_{14}AT$. Out of 12 genes, three are pseudo. Messenger RNAs coding for UGT1A7-UGT1A9 are expressed mainly in colon, small intestine, bladder, skeletal and heart muscle; UGT1A10 is only in heart muscle. The UGT1A8 isoform glucuronidates primarily complex 4-hydroxy substituted flavones and anthroquinones and certain types of 2, 4 substituted phenols. Missense mutations have been uncovered in the UGT1A6 phenol isoform. Some 33/100 individuals randomly selected are heterozygous for both a T181A and R184S mutation in one allele of the UGT1A6 gene. Complex alkyl phenol turnover by the UGT1A6variant is reduced 50%. Similarly, heterozygous promoter mutations were uncovered in the genome of CN-I and CN-II individuals; data showed both a TA insertion and a TA deletion at the TATA box of the UGT1A1 (HUG-Br1) bilirubin transferase gene. Transcriptional activity of a reporter gene fused to the A1 upstream region containing either an insertion or deletion was inhibited 80% and 35%, respectively. Additionally, the missense mutation in the CN-1 (R336W) or CN-II (I294T) patient was completely or partially inactivating, respectively. Computer analysis showed a conserved and highly hydrophobic region in the bilirubin isoform between residues 161-180; it is designated a buried structure, likely helical, centered around a 170/171 di-phenylalanine. Mutant proteins with Phe-170 changed to Leu, Val, Ala, Tyr, or Glu had 0-10% normal activity at pH 6.4, but 14 to 65% at pH 7.6. The same mutations at Phe-171 revealed 38-51% normal activity at pH 6.4 and even higher values at pH 7.6; Leu-171 was completely normal. Either Ile-170 or -171 was totally inactive at pH 6.4 with 20% activity at pH 7.6. Although certain 170 substitutions satisfied the hydrophobicity requirement, Phe-170 was not replaceable.

Project Description:

Objectives:

The primary objectives of this project are:

To carry out chromosomal walking upstream of the original version of the *UGT1* locus to select overlapping cosmid clones in order to locate other exons 1 encoding additional mRNAs with the 3' common exons at this locus.

To carry out restriction enzyme and sequence analyses of cosmid clones which overlap and extend the *UGT1* locus.

To determine the defects among as many Crigler-Najjar, Types I and II, and Gilbert's individuals, as possible.

To use the conformational polymorphisms (SSCP) for rapid screening of Crigler-Najjar individuals and Gilbert's individuals to locate defects at the *UGT1* locus.

To use PCR-amplification and PCR-sequencing to define specific alterations uncovered by the SSCP technique.

To make missense mutations in the normal bilirubin or phenol transferase cDNAs (isolated in our laboratory) consistent with each missense mutation uncovered in the genome of patients with defective bilirubin/phenol glucuronidation.

To express individually mutated bilirubin transferase cDNAs in COS-1 cells either transiently by using recombinant plasmids or a stably expressing Hep.G2 cell line using recombinant vaccinia viral constructs.

To determine the structure-function relationship of each mutation of bilirubin transferase activity by using a ^{14}C-labeled substrate in an *in vitro* assay which separates each of the three bilirubin glucuronides or by increasing the sensitivity by developing and analyzing glucuronides on a HPLC system.

To use biochemical techniques to analyze a unique hydrophobic region and a protein β-loop in the major bilirubin isoform for effects on glucuronidating activity.

To isolate cDNAs and characterize the encoded protein for all viable new exons 1 uncovered at the UGT1 locus, testing a range of therapeutic and nontherapeutic chemicals.

To purify the baculoviral ecdysone UDP-glucosyltransferase (egt) using a recombinant egt cDNA-viral construct supplied by a collaborator (Dr. D. R. O'Reilly) for expression in either the SF9 insect cell, a bacterial GST gene fusion system, or the pPIC9 gene fusion system for expression in the *Pichia pastoris* yeast system.

To use the egt protein in crystallographic studies (carried out in collaboration with Dr. J. Jones) to determine its x-ray diffraction pattern for tertiary structural analysis.

To carry out computer modeling of the crystallographic structure of the egt protein and to derive a conformation for the analogous bilirubin UDP-glucuronosyltransferase using the results from our structure-function relationship studies.

To use the flanking regions from the major bilirubin and critical phenol transferase genes contained in the *UGT1* complex in connection with a reporter-gene to locate regulatory elements.

To carry out deletions and/or site-directed mutagenesis to establish precise regulatory nucleotide sequences to be used in connection with the detection of nuclear regulatory factors.

To isolate and use mRNA from liver and kidney of variously treated monkeys (provided to us by a collaborator) to establish whether any of the species encoded at the *UGT1* complex is inducible and, if so, by which effector compounds.

To construct vectors containing retroviral promoter elements/enhancers containing the HUG-Br1 or the HUG-Br2 cDNA for the stable expression of bilirubin transferase activity in Hep.G2 cell.

To make specific synthetic peptides of the two bilirubin transferase proteins for the development of monoclonal antibodies.

To determine mRNA tissue distribution for the newly uncovered *UGT1*-encoded transferase isoforms to explore the physiological consequences of isoform distribution and, thus, function.

Methods Employed:

The principal methods employed are: 1) Enzyme assays using spectrophotometric, spectrophotofluorometric, and radiometric determinations of products separated by TLC chromatography; 2) HPLC Chromatography; 3) Cloning of cDNAs into phage and plasmid vectors; 4) Sublconing of cDNA and genomic DNA fragments; 5) Polymerase chain reactions to synthesize ample quantities of specific DNA; 6) Single-strand and double-strand DNA sequencing using the PCR technique; 7) Computer analysis of nucleotide sequence data; 8) Agarose gel electrophoresis of DNA and RNA; 9) Filter hybridization of DNA probes using Southern, Northern, slot, and dot blot techniques; 10) Site-directed mutagenesis of critical bases to test the effect of missense and selected alterations on activity; 11) Restriction-enzyme mapping of genomic DNA; 12) Isolation of DNA inserts in plasmid and phage by endonuclease treatment, extraction, and centrifugation; 13) Transformation of yeast and transfection of COS-1 and Hep.G2 cells with expression vectors containing human transferase cDNA inserts; 14) Tissue culture techniques; 15) Western blot analysis of transferases; 16) Monoclonal antibody production using synthetic peptides based on deduced amino acid sequences derived from cDNA sequence data; 17) Immunoprecipitation of transferase-specific proteins directed at specific determinants including possible acceptor substrate, as well as donor substrate, binding sites; 18) construction of target DNA-reporter gene fusion systems for analysis of nucleotide regulatory elements in genes; 19) transfection studies of target DNA-reporter gene fusion constructs into appropriate cell lines; 20) construction of deletion mutants to locate regulatory nucleotide sequences; 21) Purification of enzyme protein by cation exchange chromatography and gel filtration; (22) computer modeling of the tertiary structure of the related baculoviral UDP-glucosyltransferase to derive a conformation of the human bilirubin isozyme.

Major Findings:

Our description of the *UGT1* locus is now essentially complete. Its description has been critical to determining defects in patients with Crigler-Najjar(CN) diseases. The original version of the gene complex, UGT1A-UGT1F, codes for bilirubin, bilirubin-like, and phenol UDPglucuronosyltransferases. In the 5' region of the locus, six different exons 1, each with an upstream promoter and each encoding the amino terminus of an isoform, are arrayed in series with 4 common exons encoding six identical carboxyl termini in the 3' region. We have extended the locus by identifying, sequencing, and mapping six additional exons 1 coding for UGT1A7-UGT1A12P genes. The most 5'exon was used to select other cosmid clones to extend the locus by more than 310 kb. The total size of the locus is more than 410 kb: 95 kb for the original version and a minimal of 350 kb for the extended portion. The six additional exons 1 are contained in 10 cosmid clones. The restriction endonuclease-mapping and Southern blot hybridization analysis are nearly complete. The six exons have been sequenced and arranged in a series. Due to a high degree of identity for the last six exons and, thus, all exonic probes, jumping along the locus contributed to gaps. Except for one, we have created overlap among the cosmids using unique intronic regions as probes obtained from the ends of each cosmid insert in conjunction with Southern blot analysis. The nucleotide data at the ends of each insert were obtained by sequencing from the cosmid vector itself.

The novelty of this locus has provoked confusion among the Human Nomenclature Committee and others as to an appropriate designation: a single gene or multiple genes. An agreement has been reached that the locus represents multiple genes of the UGT1 family of transferase isozymes with each having independent regulation but linked through the 3' end. To be consistent with the nomenclature for other transferase families and in agreement with other researchers in the field, my laboratory has taken the lead in renaming the UGT1 gene complex. The previous designation of the genes UGT1A through UGT1F in this document and publications will be renamed in turn, UGT1A1 through UGT1A6. In the extended portion of the locus, newly identified exons 1 representing genes continue with the nomenclature as follows: UGT1A7 through UGT1A12.

UGT1A7 through UGT1A12 is a cluster of exons 1 with 86 to 91% sequence identity which, no doubt, represents a subfamily within the UGT1 family. Due to at least six nucleotide deletions scattered through out each of the exons 1 of UGT1A11 and UGT1A12, only four of the last six exons 1 are predicted to encode a viable mRNA. Hence, UGT1A11P and UGT1A12P are pseudo genes. Except for UGT1A12P, a TATA box element is located between positions -137 to -121 bp upstream of each exon 1 in this cluster. The element for UGT1A7 through UGT1A10 is: AAT_9AAA or $AT_{14}AT$ and that for UGT1A11P is AT_8G_6T. Presumably, six thymidines were converted to six guanidines in the UGT1A11P element which are likely functioning as a G-C clamp to render the pseudo gene transcriptionally inactive. Based on nucleotide alignment of the highly similar upstream regions of the UGT1A7 through UGT1A11P genes, nucleotides from -40 to -178 bp upstream of exon 1 of UGT1A12P have been deleted.

The tissue distribution of mRNA encoding UGT1A7 through UGT1A10 was determined with exons 1-specific probes. Each of the messenger RNA species encoding UGT1A7 through UGT1A9 is located primarily in the colon, small intestine, bladder, sheletal muscle, and heart. The UGT1A10 gene is expressed only in heart muscle. UGT1A9 is also distributed in the typical glucuronidating organs, liver and kidney.

Since bilirubin is an acyl-glucuronide, it is expected that some of the newly uncovered exons at this locus will encode transferases involved in acyl glucuronidation and idiosyncratic metabolism of certain therapeutic drugs, including the nonsteroidal anti-inflammatory drugs (NSAIDS). In certain subpopulations of individuals unidentified UDP-glucuronosyltransferase(s) form the highly reactive acyl glucuronides of certain NSAIDS and other chemicals which undergo rearrangement to generate "leaving groups". Such groups attack and covalently bind to proteins forming haptenes and, subsequently, the development of severe autoimmune responses. The acyl bilirubin-glucuronide rearranges but does not form reactive leaving groups. The extent of overlapping substrate specificity of the bilirubin isoform(s) for these types of drugs or the substrate specificity of these new isoforms remains to be determined.

In order to expedite the determination of substrate specificity of these newly uncovered and encoded isoforms, we constructed the equivalent cDNAs for expression in COS-1 cells. Since a cDNA, HLUG P4, encoded by UGT1A9 had already been reported in the literature, we isolated the same cDNA from a hepatocyte cDNAλZAP library to use in the construction of cDNAs representing the UGT1A7, UGT1A8 and UGT1A10 genes. Overlaps between the exon 1 of each of these genes and a common fragment from the UGT1A9cDNA were made using polymerase chain reaction-amplified material. The overlap was extend to generate the cDNAs, each with its respective unique 5' terminus. The UGT1A9 cDNA contains some 60 nucleotides of common sequence present at the 3' end of exons 1 of each of the UGT1A7-UGT1A10 genes; the 60 nucleotides are contiguous with the identical 3' common end (738 nt) for all genes encoded at this locus. Hence, the 798 bp-3' end of the UGT1A7 through UGT1A10 cDNAs are predicted to be identical. These simple constructions of the cDNAs was required since we were enable to select these particular cDNAs from a human hepatocyte cDNAλZAP library. Two cDNAs representing exons 1 of UGT1A3 and UGT1A5 uncovered during the initial characterization of the UGT1 locus have been constructed in a similar fashion using the cDNA for UGT1A4 (HUG-Br2). All cloned isoforms are undergoing analysis for substrate specificity.

The UGT1A8 cDNA was expressed in the COS-1 cells and analyzed for glucuronidation using 11 different categories of acceptor substrates. The rank in substrate turnover was flavones > anthroquinones > 17α-ethynlestradiol > complex alkyl phenols > biphenyls > bilirubin> benzo[a]pyrenes >α-naphthol. Results for the two best categories, flavones and anthroquinones, indicate that this isoform preferentially glucuronidates compounds with complex three-ringed structure substituted with hydroxyl groups. Flavones with 5-hydroxyl groups inhibit growth of gastric and colon cancer cells. Similarly, the UGT1A8 isoforms requires the hydroxyl group substituted at position 5 for glucuronidation.

We have uncovered for the first time missense mutations in exons at this locus which code for phenol-selecting domains. Exon 1 of the UGT1A6 (UGT1F) gene encodes the acetaminophen-selecting domain of the isoform. We have uncovered two missense mutations in one allele of the UGT1A6 gene of an individual, creating heterozygosity. The mutations, T181A and R184S, created BsmI and BsoFI endonuclease sites at the two respective mutations. We have verified heterozygosity by BsmI- and BsoFI-sensitivity of PCR-amplified genomic DNA from this individual. Upon screening some 98 individual DNA samples, we uncovered 33 individuals who were also heterozygous for the two mutations on one allele. For one individual, in addition to the double mutation on one allele, the R814S mutation was on the second allele. Any manifestations of drug reactions for this individual could not be ascertained. It is predicted that 2/100 individuals are homozygous for the two linked mutations. We carried out acceptor substrate activities with the UGT1A6 and the UGT1A6variant (A+ and S+) isoforms using 16 chemicals from 6 categories. It was uncovered for the first time that both isozymes glucuronidate 1.1 to 6-fold better at pH 6.4 than at the typical pH 7.6 assay condition. Among the extensive number of compounds and types of compounds an isoform can metabolize, the rank of chemical turnover for the UGT1A6 isoform was: simple phenols> coumarins > complex alkyl phenols > α-naphthol > acetaminophen. While the UGT1A6variant metabolized the coumarins and the α-napthol similar to that by the wild type enzyme, metabolism of the complex alkyl phenols and acetaminophen were reduced 50% and 25%, respectively. Analysis of the data show that while size and bulkiness are factors which restrict glucuronidation by the isoforms, the pKa value of the hydroxy group of all substrates was 7.8 and above. The UGT1A6 protein and its rat homolog were previously shown by other researchers to glucuronidate acetaminophen-- an analgesic causing extreme sensitivity in certain individuals. (The Gunn rat which carries a mutation in a common exon at the UGT1 locus is 100-fold more sensitive to high doses of acetaminophen and succumb to such treatment, unlike the normal Wistar rat.) This human UGT1A6 variant, however, showed only a 25% reduction in, turnover of the chemical.

The characterization of the UGT1 locus, encoding multiple bilirubin transferase isozymes, allowed us to uncover 13 different mutations in the UGT1A1 gene encoding the major bilirubin transferase isozyme of Crigler-Najjar (CN) Type I and II patients. Of the eight missense mutations uncovered, we have shown that the 4 seen in CN-I patients completely inactivated the major bilirubin transferase activity at pH 6.4 and that the 4 seen in CN-II patients only partially inactivated the enzyme accounting for residual activity in CN-II patients.

A new class of mutations at the UGT1 locus involving the TATA box transcriptional element of the UGT1A1 gene was found as heterozygous genotypes for both CN-I (CF) and CN-II (SM/TS) individuals; each patient contained a second missense mutation in the coding region. An allele contained either a TA deletion or TA insertion at the natural, ATA_7A, TATA box element of both the CN-I and the CN-II patients. With the pCAT reporter gene fused with the A1 upstream regulatory region (-1.7 kb) containing either a wild-type $[A(TA)_7A]$, a TA deleted $[A(TA)_6A]$, or a TA inserted $[A(TA)_8A]$ at the TATA box, expression in HepG2 cells showed that the transcriptional rates were 65% and 20% normal, respectively, for the TA_6 and TA_8 mutants. As stated all patients were compound heterozygotes. In the coding region, the mutations I294T (SM), M310V(TS), R336W(CF), and I431T(TS) reduced glucuronidation rates to 22%, 40%, 3%, and 57% normal. From these *in vitro* results, it is predicted that the level of bilirubin transferase contributed by the two alleles of: CF (CN-I), SM (CN-II), and TS (CN-II) is 11.5%, 21%, and 48% normal, respectively.

The evidence presented here on CN diseases and recently published data on Gilbert's individuals show that a single genetic defect (TATA box mutations) can exist between the CN diseases and the Gilbert's syndrome. Hence, the severity of the phenotypes depends upon the additive effect of at least two mutations whether homozygous or compound heterozygous. Together the results support the conclusion that these clinical phenotypes are a manifestation of a spectrum of mild to deleterious mutations (genotypes) among the three population of individuals. The inheritance of all the bilirubin-related diseases, previously considered unclear, can be accounted for by recessive inheritance of combinations of defects at the UGT1A1 gene culminating in the phenotypes (CN-I, CN-II, and Gilbert's) observed in the population.

Gel mobility analysis with radioactively labeled wild type or mutant TATA box oligos showed that a hepatocyte-specific nuclear protein binds to $[A(TA)_6A]$ and $[A(TA)_8A]$ at low protein concentration (0.02 to 0.5 μg) but not to the wild type sequences. Higher levels of the protein(s) bound to $[A(TA)_8A]$ than to $[A(TA)_6A]$ to cause the band shift; this result parallels the level of transcriptional inhibition by the two mutant TATA box elements. Furthermore, either element can be displaced with excess nonradioactive TA_6 or TA_8 oligo in a concentration dependent manner; the wild type sequences did not displace the mutant sequences. At higher protein levels (>1.0 μg), a similar mobility shift was seen with wild type and the mutant oligos.

We continued to carry out structural analysis of the major bilirubin isoform UGT1A1 (HUG-Br1) focusing on a highly hydrophobic region (microregion A) identified by a natural mutation uncovered in a CN-I patient. A phenylalanine deletion at codon 170 in exon 1 of UGT1A1 disrupted a conserved di-phenylalanine among the bilirubin/bilirubin-like (UGT1A1-UGT15) isoforms. Studies with mutant UGT1A1 derived from the pΔF170HUG-Br1 cDNA expression unit transfected into COS-1 cells demonstrated pH sensitivity with respect to generating product; at the routinely used pH (7.6) for bilirubin glucuronidation both the mutant and wild type proteins had similar activities. UGT1A1 isoform showed 3-4 fold more activity at pH 6.4 than at pH 7.6, and the mutant had no activity at pH 6.4. The pH dependency of human liver microsomal bilirubin transferase activity was similar to that of UGT1A1 protein.

Using the RAO-ARGOS computer program designed to detect buried helices in membrane-anchored proteins, we discovered that the membrane-bound UGT1A1 protein contains a buried helix in microregion A, a structure shown to be unique to bilirubin isoforms. Peak hydrophobicity centers around the 170/171 di-phenylalanine. Prompted by the unique hydrophobic characteristics of this region in the UGT1A1-type proteins, we made select amino acid changes in this region and analyzed for structure-function relationship of the substitutions. Depending upon the amino acid in the 170/171 di-phenylalanine and of other hydrophobic residues in the region, the RAO-ARGOS program reflected effects on the buried helix. Mutant proteins with Phe-170 changed to Leu, Val, Ala, Tyr, or Glu had 0-10% normal activity at pH 6.4, but 14 to 65% normal activity at pH 7.6. Mutant proteins with Phe-171 changed to Leu, Val, Ala, and Tyr revealed 38-51% normal activity at pH 6.4 and higher values at pH 7.6; Leu-171 was completely normal. Ile- at 170 or 171 was totally inactive at pH 6.4 with 20% activity at pH 7.6. Although certain 170 substitutions satisfied the hydrophobicity requirement, Phe-170 was not replaceable. No doubt Phe-170 is critical for the appropriate helical structure in this region. This hydrophobic region-- critical for bilirubin transferase activity-- reflects the most hydrophobic milieu in the protein, presumably, for binding/interacting with the extremely hydrophobic-behaving bilirubin substrate.

Since all mammalian UDP-glucuronosyltransferases are endoplasmic reticulum-bound and, thus, difficult to purify for crystallography studies, a soluble surrogate protein would represent a alternative strategy for obtaining information on the active site of the UDP-glucuronosyltransferases. The conservation of residues between the distant and related soluble baculoviral ecdysone UDP-glucosyltransferase (egt) and the UDP-glucuronosyltransferases suggests that this baculoviral enzyme could serve as a protein model for tertiary structural studies in lieu of mammalian UDP-glucuronosyltransferases. The viral protein catalyzes a similar reaction as the mammalian enzymes; it conjugates glucose, donated by UDP-glucose, to ecdysone which inactivates the insect hormone. There is the strict conservation of a glycine-glycine di-peptide in all UDP-glucuronosyltransferase and the baculoviral transferase as well as 61%/21% sequence similarity/identity within the equivalent common-end segment (excluding the final 30 amino-acid for membrane-anchoring). We have proceeded with adapting the UDP-glucosyltransferase as a model for crystallization studies as a strategy to gain information on the active site of the human bilirubin transferase. We are attempting to purify sufficient quantities of the enzyme for crystallography studies. We were able to increase the synthesis in the baculoviral system by a modest 2 to 3 fold when our collaborator, Dr. O'Reilly, relocated the open-reading-frame behind the baculoviral polyhedros promoter. The viral protein expressed in bacteria was inactive, due most likely to the lack of glycosylation at some or all of the nine potential sites in the protein. We will attempt expression in the Pichia pastoris yeast system.

Significance to Biomedical Research and the Program of the Institute:

Drug oxygenations and subsequent glucuronidations are catalyzed by a endoplasmic reticulum-bound multistep enzyme complex. These enzymes are important in the process of detoxification and elimination of many normal body constituents and many foreign chemicals taken in from our environment in the form of dietary food, food additives, air pollutants, and medication. There are, as well, diseases of humans ascribed to defects (not well understood) in drug-conjugating enzymes. Examples in pediatrics include neonatal hyperbilirubinemia, Gilbert's syndrome, and the Crigler-Najjar diseases. Bilirubin, one of the most toxic metabolites in the body undergoing constant production from the normal turnover of red blood cells is completely dependent upon the UDP-glucuronosyltransferase for its detoxification. Unconjugated, the heme derivative is a potent neurotoxin which leads to jaundice and kernicteric (brain) injury.

Furthermore, there are drug interactions in which the administration of two or more drugs concurrently may alter the half-life and, thus, the pharmacological effect of one of the drugs. This type of alteration in drug half-life has often been ascribed to competition of substrates at the site of glucuronidation. The extension of a drug half-life can be critical when there is a narrow margin of safety. Idiopathic drug reactions are also attributed to differences in glucuronidation rates between patients; however, there is no verification that glucuronidation plays a role.

Teratogenesis and neonatal drug toxicities are proposed to occur as a consequence of deficient glucuronidation with critical drugs, e.g., dilantin and chloramphenicol. Further, impaired/absent dihydrotestosterone glucuronidation has been associated with benign prostatic hyperplasia (BPH)/prostate cancer, respectively.

The recent isolation and characterization of the bilirubin transferase gene are important to the detection of the nature of the defective allele(s) from the Crigler-Najjar Types I and II patients and from Gilbert's individuals whose alteration(s) is also inherited. With the isolation of the gene we will be able to compare replacement of bilirubin transferase activity in the Gunn rat model with the gene, as well as the cDNAs (also isolated in our laboratory). If we are successful in establishing a Crigler-Najjar cell line and restore bilirubin transferase activity in Gunn rat, these replacement experiments can be repeated in the human mutant cells. Research which contributes to the improvement or elimination of the neurotoxicity of bilirubin and the associated emotional stress in youths caused by the jaundiced skin tones represent a primary goal of the Institute to improve the health of children. This work will also lead to therapies for defective bilirubin metabolism.

To the contrary, recent studies have demonstrated that the acyl-group of certain glucuronides of NSAIDS can migrate creating a reactive "leaving group" that can covalent attacked endogenous molecules, particularly proteins, leading to haptenes and the development of autoimmune responses with lethality in some individuals. This would represent a toxification model for glucuronidation.

Potentially, we have the opportunity to identify and characterize new transferase isoforms; some promise to be toxifying (acyl-glucuronides) as well as detoxifying (acetaminophen). The results from such studies will lead to new considerations in drug development with respect to potential toxins.

Preliminary results show that UGT1A8, as a member of the UGT1A7-UGT1A10 cluster, to be very active toward metabolizing complex flavonoids and anthroquinones; flavonoids are anti-cancer agents. Humans ingest these chemicals as a component of plants in the diet. The significance of these catalysis is not yet understood. It should be pointed out that the mRNAs encoding this cluster of enzymes are located primarily in the colon, small intestine, bladder, cardiac and skeletal muscle compared with the typical distribution of most transferase activity in liver and kidney.

Research findings which identify the human transferase cDNA encoding the enzyme responsible for glucuronidating and maintaining optimal concentrations of the critical bile salt in humans constitute a major advancement. Bile acids are important in the emulsification of fat in the diet, but excess hyodeoxycholic acid in serum and in the bile is associated with cholestatic liver disease. Since we have shown that phenobarbital induces the mRNA encoding this isoform, this or similar agents can be tested as therapeutic agents to counter the progression of cholestasis. The demonstration that the transferase which glucuronidates the bile acid also glucuronidates estriols and 4-hydroxy catechol estrogens provides a basis for understanding possible interactions between estrogen metabolites and the bile acid.

The recent characterization of a human dihydrotestosterone transferase cDNA including its location in target tissue, testis and prostate, as well as liver provides a model for the determination of the relationship between BPH and other prostate hyperplastic diseases and failures in dihydrotestoserone glucuronidation.

Proposed Course:

To determine by using bilirubin transferase-specific cDNA probes and designated restriction enzyme sites if the RFLP technique can be used to show allelic differences in Crigler-Najjar patients compared to the normal controls, siblings, and parents whether such a rapid and a specific diagnosis for the range of genotypes for the disease can be established.

To adapt the SSCP method as a preliminary screen for defects in Crigler-Najjar patients and Gilbert's individuals to provide a more rapid determination of defects.

To sequence the region identified by the SSCP technique to define the specific alteration in the *UGT1* gene complex in individuals with the Crigler-Najjar, Type I and II diseases and the Gilbert's syndrome.

To develop a Gunn rat liver cell model for bilirubin transferase gene replacement.

To collaborate with investigators at the NIH on the adaptations of the *in vitro* models for *in vivo* replacement of the bilirubin transferase cDNA or gene.

To make selected mutations in the UGT1A1 and UGT1A4 cDNAs followed by expression in COS-1 cells and assesssing the effects on catalytic activity.

To use cDNA constructs encoding two other bilirubin-like transferases based on newly uncovered exons in the gene complex for expressing the respective proteins in cell culture to assess glucuronidating activity.

To establish the substrate specificity of new isoforms uncovered in the extension of the original locus.

To determine the effects of mutations in nonbilirubin UGT1 encoded isoforms uncovered using a range of therapeutic and nontherapeutic chemicals.

To study in detail the regulation of mRNAs encoding the bilirubin-and bilirubin-like transferases and that of the hyodeoxycholate acid transferase - using poly(A)$_+$RNA isolated from variously treated monkeys.

To continue to study enhancer and promoter activity (with and without appropriate inducer compounds) for the 5' flanking regions of the two bilirubin transferase genes using the gene upstream fragment-reporter gene fusion construct for transfection into the human liver Hep.G2 cell line.

To determine if other substrates are glucuronidated by the bilirubin transferases.

To establish kinetic parameters for the different transferase isoforms.

To make synthetic transferase peptides from deduced nucleotide sequence data to develop antibodies to carry out tissue distribution and localizations of isozymes especially for the human bilirubin transferase isoforms.

To collaborate in analyzing surgical prostate specimens to determine the level of mRNA encoding the dihydrotestosterone-specific transferase cDNA and disease state of the tissue.

To purify the soluble baculoviral UDP-glucosyltransferase for crystallographic studies to use as a model for a computer derived-model of the conformation of the bilirubin UDP-glucuronosyltransferase

Publications:

Chen, F, Zhou, J, Ritter, JK, Bondy, CA, Owens, IS. Phenol and bilirubin UDP-glucuronosyltransferase RNAs are evenly distributed across the human liver lobule. Gastroenterology (1996);111:472-480.

Ciotti, M, Obrary, R, Martin, MG, Owens, IS. Genetic defects at the UGT1 locus associated with Crigler-Najjar Type I disease: Including a prenatal diagnosis. Amer J Med Genetics (In Press).

Ciotti, M, Owens, IS. Evidence for overlapping active sites for 17α-ethynlestradiol and bilirubin in the human major bilirubin UDP-glucuronosyltransferase. Biochemistry (In Press).

Owens, IS, Ritter, JK, Yeatman, MT, Chen, F. The novel *UGT1* gene complex links bilirubin, xenobiotics, and therapeutic drug metabolism by encoding UDP-glucuronosyltransferase isozymes with a common carboxyl terminus. J Pharmocokinetics Biopharmaceutics (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 00404-14 HDB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell and Sulfur Metabolism in Fibroblasts of Genetic Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Jean DeBrohun Butler	Senior Investigator	HDB, NICHD
Others:	Kristin McAlister-Norten	Pre-IRTA	HDB, NICHD

COOPERATING UNITS *(if any)*

Laboratory of Developmental and Metabolic Neurology, NINCDS (P.G. Pentchev); Laboratory of Cell Biology and Genetics, NIDDK (M. Levine); Section on Developmental Genetics, HDB, NICHD (A.B. Mukherjee); Laboratory of Cellular and Developmental Biology, NIDDK (J. Blanchette-Mackie).

LAB/BRANCH

Heritable Disorders Branch

SECTION

Unit on Genetic Diseases Involving Cystine Storage

INSTITUTE AND LOCATION

NICHD, NIH, 10/10N308 Bethesda, MD 20892-1830.

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Commercially obtained cystine binding protein (CBP) of E. coli has been purified by HPLC, yielding a pure cystine binding protein not similar to any protein in the data banks. This protein has been partially sequenced, an N-terminal peptide made and an antibody to this peptide obtained which reacts with the whole protein. The commercial preparation of CBP also contains a histidine binding protein which can be used to assay for histidine in the same way that CBP is used to assay tissues for cystine. CBP of E. coli is being cloned by two methods. One, by using an E. coli expression library and identifying the correct CBP producing colony with our antibody to CBP. Two, by making oligonucleotides of sections of the known N-terminal sequence of CBP and using them as primers to identify CBP in the E. coli library. By this later method, we have obtained a partial sequence of the DNA of CBP and are are continuing the work to obtain the complete sequence. This DNA sequence will then be cloned in a suitable bacterium for large production of cystine binding protein. With this larger amount of material, the protein will be studied as to its metabolic function, specifically its cystine binding capability.

2. Niemann-Pick C disease (NPC) was shown to be reversibly induced in normal fibroblasts by hydrophobic amines and certain steroids, thus creating a model system for study of this disease. We are identifying the trafficking patterns and metabolism of intracellular cholesterol using various inhibitors and stimulators.

3. We have shown that normal and cystinotic fibroblasts take up ascorbic acid by two mechanisms, a high affinity Na^+ dependent transporter and a low affinity Na^+ independent transporter, neither of which can be accounted for by diffusion. Transport of ascorbic acid by the low affinity transporter is not different from normal in the two types of cells, however transport in cystinotic cells by the high affinity transporter is about 40% of that seen in normal cells. The Km's for both transporters were the same for both types of cells while the Vmax of the high affinity transporter was statistically lower than normal for cystinotic fibroblasts. This data indicates that cystinotic fibroblasts have a selective impairment in ascorbate transport.

Project Description:

Objectives:

1. To elucidate the basic biochemical defect in genetic diseases, specifically cystinosis and Niemann-Pick C & D.

2. To explore secondary biochemical and physiological derangements caused by the primary metabolic defect.

3. To develop new and improved methods of treatment and diagnosis.

4. To use genetically abnormal cells along with normal cells to understand human basic biochemistry.

Methods Employed:

1. Cell tissue culture techniques.

2. Spectrophotometric, radiochemical, and fluorometric enzyme assays.

3. Ultracentrifugation and cell fractionation techniques.

4. Radioisotope measurements

5. Light and phase contrast microscopy.

6. Column, thin layer and high performance liquid chromatography.

7. Gel and high voltage electrophoresis.

8. Immunochemical techniques.

9. Molecular Biology techniques.

Major Findings:

1. Commercially obtained cystine binding protein of E. coli is heterogeneous. Purification gave four proteins. One protein bound cystine and was not similar to known proteins in data banks. Another protein was highly similar to a known histidine binding protein of S. typhimurium and can be used to specifically analyze tissues for histidine content as is now done for cystine.

2. Normal fibroblasts can reversibly be induced by hydrophobic amines and certain steroids to mimic NPC disease. With this model we are studying the development of this disease and subsequent restoration to normal using known agonists and antagonists of intracellular lipid transport and metabolism. We find that trafficking of intracellular cholesterol using both biochemical and cytochemical methods is via the golgi.

3. Normal and cystinotic fibroblasts have two ascorbic acid transporters, a high affinity Na^+ dependent transporter and a low affinity Na^+ independent transporter, neither of which are due to diffusion. One of these transporters is quantitatively different in cystinotic cells.

4. A mutant mouse was found which stores cystine and cholesterol in lysosomes of various tissues. This mouse is considered a model for NPC disease and now possibly for cystinosis. No animal model for cystinosis has previously been known.

Significance to Biomedical Research and the Program of the Institute:

The study of genetic inborn errors is a powerful tool for elucidation of biochemical mechanisms in normal as well as abnormal states. The discovery of formerly unknown metabolic pathways in cystinosis and in NPC disease in sulfur and in cholesterol metabolism will undoubtedly contribute greatly to our understanding of these processses in general and will lead to better methods of diagnosis, treatment, prevention and understanding of these and other disorders, and will have substantial public health significance.

Proposed Course:
Characterization of a cystinotic cystine associated protein in fibroblasts will be continued. CBP studies will be continued with the aim of finding the actual binding site for cystine in the purified CBP. Using immunochemistry and molecular biology techniques, the pure CBP of E. Coli will be cloned and proteins from eukaryotic cells will be sought with similar cystine binding properties and sites with the hope that a lysosomal cystine binding protein functioning in the egress of cystine from lysosomes will be identified.

All of the mutant diseases being studied where cystine and/or cholesterol are stored intracellularly (NPC model mutant mouse, cystinosis, I-cell disease and NPC), show a clinical degenerative neurological involvement. Cholesterol is absolutely essential for cell viability and the cellular level of this sterol is closely regulated and finely tuned. Abnormal changes in cellular cholesterol levels can have devastating effects neurologically and excess membrane cholesterol is known to inhibit transport mechanisms. The pathway for cholesterol homeostasis has been brilliantly worked out by Brown and Goldstein and is used as a model system for receptor mediated cellular uptake. However, we are finding in working with the above mutant cells that in the case of cholesterol homeostasis, additional previously unknown controls must be operating, particularly in the area of transport of cholesterol out of the lysosome to its sites of cellular regulatory responses. It has been assumed that cholesterol diffuses or is carried by a protein from lysosomes to processsing sites. NPC cells do not display this movement of cholesterol, rather free cholesterol accumulates in the lysosomal and golgi regions. We are testing these cells for lack of a normal cholesterol transport system or for the presence of an abnormal cholesterol binding system. Discovery of a way to create the NPC disease state in normal fibroblasts is very advantageous to these studies of the trafficking patterns of cholesterol in fibroblasts. The mutant mouse and I-cell disease where cystine and cholesterol are stored in lysosomes and cystinotic fibroblasts where cystine is stored in lysosomes will be similarly tested to ascertain their cholesterol regulating mechanisms. In cystinotic fibroblasts where cystine accumulates in lysosomes, there is no simultaneous accumulation of free cholesterol, however intracellular levels of stored excess cholesterol esters are low and circulating cholesterol levels in patients are high. This may reflect an abnormal cellular handling of cholesterol by cystinotic cells.

We are continuing studies on the kinetics of uptake of ascorbic acid (AA) by normal and cystinotic fibroblasts showing two transporters. Ascorbic acid lowers the cystine content of cystinotic fibroblasts by unknown mechanisms. The knowledge that the Vmax of uptake of ascorbic acid by cystinotic cells is normal for the low affinity transporter and deficient for the high affinity transporter, indicates a need to explore the intracellular use of this vitamin in these mutant cells.

We are also investigating alternative ways of depleting cystine and/or cholesterol in fibroblasts and in mouse tisses since there has not previously been an animal model for the above diseases.

Publications:

Blanchette-Mackie E J, Coxey R A, Butler J D, Roff C F, Campbell G, Dwwyer N K, Carstea E D, Goldin E, Dawidowicz E A, Brady R O and Pentchev P G The Intracellular Distribution of Cholesterol can be Modulated at Lysosomes, the Golgi Complex and the Rough Endoplasmic Reticulum. Proc. Nat. Acad. Sci. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00408-13 HDB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Heritable Disorders of Connective Tissue

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Joan C. Marini, M.D., Ph.D.	Senior Investigator	HDB, NICHD
Others:	Isabelle Cohen, Ph.D.	Senior Staff Fellow	HDB, NICHD
	Antonella Forlino, Ph.D.	Fogarty Fellow	HDB, NICHD
	Gabriele Grassi, M.D., Ph.D.	Fogarty Fellow	HDB, NICHD
	Anna Gajko, Ph.D.	Special Volunteer	HDB, NICHD
	Paul Dawson	Visiting Associate	
	Wayne Cabral	Biologist	Contract Technician

COOPERATING UNITS *(if any)*

Dept. of Rehabilitation Medicine, CC (N.L. Gerber); Laboratory of Developmental Endocrinology (G. Chrousos); Laboratory of Mammalian Genes & Development (H. Westphal); Sect on Metabolic Analysis & Mass Spectrometry (A. Yergey); Genetics Unit, Shriners' Hospital, Montreal (F. Glorieux)

LAB/BRANCH

Heritable Disorders Branch

SECTION

Connective Tissue Disorders

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.5	5.5	1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Section has continued its studies aimed at elucidating the molecular mechanisms of heritable disorders of connective tissue, specifically osteogenesis imperfecta (OI) and Ehlers-Danlos (EDS), and at applying this information to the treatment of these disorders.

One continuing interest of the Section is to identify the collagen mutations in patients with OI and EDS and determine the relationship between the type and location of the mutation and the severity of the connective tissue disorder. Mutations in the α2(I) collagen chain identified by this Section and other labs have provided additional support for the regional model we have proposed.

There are three primary projects in the Section. One primary project is to develop selective antisense suppression of the mutant collagen allele as an approach for therapeutic intervention. This year we have turned to the use of antisense hammerhead ribozymes. *In vitro*, we have demonstrated allele-specific cleavage and determined the parameters for cleavage. We have conducted competition experiments with total RNA and synthetic targets. We are now pursuing vector constructs to determine suppression stability and efficiency in cultured cells.

The second primary project is the generation of a murine model for non-lethal OI. We have used site-directed mutagenesis to produce a construct with a point mutation mimicking non-lethal human OI. The cre-lox recombination system will be used to control the timing of expression of the mutation and a lox-stop-lox construct has been introduced into the intron prior to the exon containing the disease-causing mutation. We have isolated recombinant ES cells and have successfully generated chimeras.

A third major focus of interest which we have been developing is in the bone biology of OI. We are using cultured osteoblasts to study the way bone cells modify and secrete mutant collagen. We are also pursing the secondary non-collagenous abnormalities of OI matrix and the response of OI osteoblasts to TGF-β stimulation.

In clinical studies, we are continuing our treatment trial of growth hormone in short children with OI to determine its effects on growth stimulation, bone density and bone morphometric properties. We are continuing our collaborative interests in the neurological aspects of OI and in maximizing the physical functioning of OI children though aggressive rehabilitation.

Project Description

Objectives:

To study the molecular biology and pathophysiology of genetic disorders of connective tissue and to use the findings of these studies for diagnostic and therapeutic intervention.

Methods Employed:

Culture of human fibroblasts, osteoblasts and chorionic villi; selective labelling of collagens and proteoglycans; standard methods of protein purification; protein analysis by SDS gels, gradient gels, cyanogen bromide cleavage of peptides; methods of molecular biology such as preparation of plasmids, subcloning, restriction enzyme mapping, riboprobe generation, coupled transcription/translation, isolation of total RNA, RNase cleavage of RNA/RNA and DNA/RNA hybrids, site-directed mutagenesis, sequencing, cDNA screening, amplification by polymerase chain reaction (PCR) of genomic and cDNA, single strand conformational polymorphism analysis (SSCP), allele specific hybridization, antisense suppression, Southern and Northern hybridizations.

Major Findings:

1) As an approach for therapeutic intervention in OI and other dominant negative disorders, we are investigating the effectiveness and selectivity of antisense suppression of the mutant collagen chain using lipid-mediated transfection. We have turned our focus to the use of antisense hammerhead ribozymes to increase both specificity and efficiency. We first demonstrated the allele specificity of the hammerhead ribozymes *in vitro*. We selected two unrelated patients whose naturally occurring mutations are responsible for the formation of a new ribozyme cleavage site in the mutant allele. In one case the mutant allele contained a GUC cleavage site; in the second case, the mutation created two new cleavage sites, GUU and UUC. For each patient, a cDNA fragment from both normal and mutant alleles was cloned into pGEM vector and mRNA was generated by *in vitro* transcription.

Increasing amounts of ribozyme, ranging from a Rz/substrate ratio of 1:1 to 1:125, were incubated with the synthetic transcripts, products were separated on denaturing gels and quantitated by densitometry or direct counting. In all cases, the ribozyme was absolutely specific for the mutant allele. This is the first demonstration that ribozymes selectively cleave mRNA molecules carrying a naturally occurring point mutation causing a genetic disorder.

In all cases, the cleavage activity depended on the time, temperature of incubation and on the ratio ribozyme/substrate. For the GUU and UUC cleavage sites, all experiments were also performed using synthetic RNA target of progressively increasing length, from about 500 to 1000 nt. The increasing substrate length did not significantly affect the rate of ribozyme cleavage. This feature is encouraging for the *in vivo* activity of ribozymes, which must involve cleavage of full length mRNA.

We also studied the rate of cleavage by ribozymes when the target synthetic RNA was mixed with different amounts of non-specific RNA or normal allele RNA. The rate of cleavage by ribozymes was not affected by the presence of total RNA up to a 500:1 weight ratio of total RNA to mutant target. The rate of cleavage is decreased approximately 50% by the presence of normal allele synthetic target at a ratio of 1:1 mutant/normal substrate. This demonstrates that binding of the complementary arms to the non-cleavable target has a slow release time *in vitro*. The binding can be destabilized by introducing a single mismatch into the middle of one of the complementary arm of the ribozyme. Under these conditions, even a 16 fold molar excess of synthetic non-cleavable substrate does not decrease the RZ cleavage rate.

2) We are pursuing our goal of generating a non-lethal murine model for OI, in which the causative collagen mutation produced a glycine substitution in α1(I) of the type which is typical for severe human OI. We used gene targeting and homologous recombination in embryonic stem cells to introduce a mutation into the mouse genome. By site-directed mutagenesis on a subcloned murine COL1A1 fragment, we changed a G → T in exon 23 at nt 1546. This mutation was previously characterized in our lab in a case of human type IV OI and causes the substitution gly349 → cys. A C → T transition at nt 1566 and a C → G transversion at nt 1568 were also generated to change Leu356 → Met to enable the detection of the mutant protein by CNBr digestion. In addition, we generated a GUC cleavage site for hammerhead ribozyme cleavage of the mutant allele mRNA by changing a C → G at nt 1551.

Since the severe OI phenotype could compromise the reproductive success of the affected mice, we will suppress the expression of the mutated allele in the first generation. To do this, we have inserted a lox-stop-lox transcription termination cassette into intron 22, which is located upstream of the exon containing the disease-causing mutation.

We electroporated the linearized construct into embryonic stem cells derived from the 129Sv murine strain. Thirteen recombinant clones were selected using a double selection with neomycin and gancyclovir. We microinjected three recombinant ES clones into recipient C57BL/6 blastocysts, which were then inserted into foster mothers. From this first round of microinjection, we have obtained two male mice which appear to be chimeras on the basis of coat color.The murine portion of this work has been done in collaboration with the laboratory of Dr. Heiner Westphal at the NICHD.

3) We have continued our investigations of OI osteoblasts cultured from the iliac crest biopsies of osteotomy chips of OI patients. Our goal is to gain insight into the molecular and biochemical mechanisms underlying bone pathophysiology. Alterations in the structure of one or more components of bone matrix or in the ratios of the various components of matrix could disrupt cell-matrix interactions, as well as the responses of osteoblasts to growth stimulants.

We have electrophoretically analyzed the type I collagen synthesized by OB and fibroblasts in 10 patients affected with type IV OI. Most OB collagens displayed a dramatic shift of the electrophoretic baseline of the collagen chains or a broader α1(I) band, whereas fibroblast collagen from the same patient displayed milder electrophoretic delay. Using cells from a patient with a novel α1(I) gly 352 →cys substitution, collagen synthesized by FB and OB in the presence of α,α'-dipyridyl co-migrated, demonstrating that the electrophoretic abnormality was post-translational in origin. Also, there is a Tm difference between this patient's FB and OB collagen. These data suggest that cellular processing of mutant collagen protein differs between OI FB and OB.

We treated the OB from an OI proband carrying an α1(I) Gly 222 → Ala substitution and an age-matched control with 5 ng/ml of TGF-β for up to 48 hrs. OI expression of matrix components was detected by Northern blot analysis of OB RNA. The mRNAs for both of the type I collagen chains were up-regulated by TGF-β after 4-48 hrs of treatment. Collagen stimulation in the OI osteoblasts was approximately half that obtained in control cells. The most dramatic changes were detected in the expression of the proteoglycans decorin and biglycan. In cells treated with 5 ng/ml TGF-β, decorin mRNA was up-regulated 4-fold in control OB. In OI OB, only a transient increase was detected, which was significantly less ($p<0.04$) than the control response. Biglycan was up-regulated more than 6-fold in control OB and less than 2-fold in OI OB ($p=0.25-0.05$). These data suggest that OI OB may be generally less responsive to TGF-β and similar experiments are being conducted in other OI osteoblasts.

We have also continued to expand our supply of frozen OI osteoblast lines. This year we have cultured osteoblasts from about 20 patients with types III and IV OI.

4) We have previously identified a unique deletion in one allele of the α1(I) gene in a family with

dominant type III OI and a pedigree suggestive of germline mosaicism. The deletion extends from the last three nucleotides of exon 33/34, through exons 35 and 36 and ends in intron 36, leaving 156 nt at the 3'-end of intron 36.

We have now demonstrated that this mutation results in three alternatively spliced forms of the mRNA from this allele. The use of different donor sites and the exon 37 acceptor site results, respectively, in (1) the loss of the last 5 nt of the deleted exon 33/34 and an out-of frame protein with early chain termination, and (2) skipping of the full exon 33/34 and a protein which is in-frame but missing exons 33-36. There is also a form of the mRNA with retention of the 3'-portion of exon 36, which would generate an in-frame protein with a non-collagenous insert. By RNA protection assay, we found that 2-7% of the transcript was in the retained intron form, 35-40% used the out-of-frame cryptic donor in exon 34 and 55-65% of the mRNA was in-frame.

SDS-PAGE of type I procollagen produced by cultured osteoblast showed normal migration of the proα(I) chains and a higher proportion of type III procollagen in the proband than in the control. Thus, the effect of the partial null allele generated by the out-of-frame message probably moderates the more severe and lethal phenotypes caused, respectively, by the retained intron and the in-frame transcripts. Furthermore, the increased α1(III)/α1(I) ratio, which is characteristic of the mild form of OI, could also moderate the lethal phenotype previously associated with the presence of large deletions in COL1A1 or COL1A2 by influencing the fibril diameter and structure.

5) We have continued to investigate the collagen protein abnormalities and associated molecular defects in patients with osteogenesis imperfecta. We have previously proposed a Regional Model for the relationship between clinical severity and mutation location. This model is based on the clustering of lethal and non-lethal phenotypes along the α2(I) chain, which suggests that the location of the mutation is the most important factor in phenotype determination for single amino acid substitutions and single exon splicing defects. The new mutations which have been delineated along the α2(I) chain are compatible in location with the regional model, in which there are now 3 lethal and 4 non-lethal regions alternating along the length of the chain. The Regional Model correctly predicts the phenotypic outcome in 90% of α2(I) mutations. This year, we have delineated a novel mutation which causes an α1(I) gly421 → asp substitution and further defines the boundary between the first lethal and non-lethal regions.

A Regional Model is also associated with phenotype for about 85% of known α1(I) mutations. However, several features of mutation phenotype in α1(I) are inconsistent with the emphasis on location of the regional model. The phenotypic outcome differs in 6 instances between exon splice defects and gly substitutions in those exons. At 8 glycine residues, substitutions of different amino acids are associated with lethal or non-lethal phenotypes.

6) One important consequence of the regional model is that it delineates which regions of the chain are crucial for collagen/extracellular matrix structure and allows further research to focus on determining the specific role(s) of the crucial regions. One possible role for crucial regions would be to engage in collagenous or non-collagenous interactions in matrix.

We have begun to purify type I collagen from several OI cases in each of the regions along the α2(I) chain for the purpose of investigating possible abnormalities in binding functions. We have now purified preparative quantities of type I collagen from 7 OI cases and normal controls and are in the process of collecting collagen from an additional 4 to 6 OI cases. Patient collagen is ammonium sulfate precipitated from the media of continuous cultures of fibroblasts after ascorbate stimulation. DEAE-cellulose 52 chromatography is utilized to remove proteoglycans. A second DEAE-cellulose 52 column is eluted with a 0-0.12 M NaCl gradient. This allows the reasonable separation of ColI pC forms, type I procollagen, type III procollagen and fibronectin. Appropriate fractions can be pooled and concentrated for further studies.

7) We have continued to study germline mosaicism in our patients. We are investigating this mechanism of inheritance of OI in both lethal and non-lethal forms of OI. This year we have continued our efforts on the three families with lethal OI. Last year, we separated the α2(I) alleles of the probands in two families; sequencing of both alleles revealed only normal sequences. The α1(I) alleles were not able to be separated by heterozygosity for polymorphisms. We have done G-screening on multiple random subclones from the target region to detect changes in glycine residues but have not identified the causative mutation. We have begun to look in the C-terminal extension of the α1(I) alleles in both families for mutations which might interfere with the rate of chain incorporation into helix.

The third case is a very interesting family in which each parent has had a normal child with another partner but together they have now had three children with the lethal form of OI and no normal children. Our examination of offspring collagen indicates that overmodification extends the full length of the helix.

8) We are continuing our interest in the molecular basis of variability of expression of the same collagen mutation. We have delineated the same α1(I) mutation, gly 352 → ser, in two families. In one family, a man with severe type III OI has two sons affected with moderate type IV OI. In an unrelated family, the mutation occurs in a girl with type III OI. Subsequently, this mutation was identified in an OI type III cell line in a cell bank. The genetic phenomenon of variable expression occurs particularly in dominant structural disorders. Of the small number of recurrences of collagen mutations which have been delineated to date, this represents the only known instance in which individuals with the same collagen mutation have dramatically different phenotypes and is thus an especially appropriate situation for further study. We have begun to examine this question by sequencing the α1(I) cDNAs for secondary mutations. In all five affected individuals, we have sequenced the entire α1(I) cDNA for both normal and mutant alleles and found no secondary mutations. One non-conservative change (Pro → Ser at aa 71) was detected in both alleles of all five individuals. We have examined the α2(I) cDNA of the probands for polymorphisms which would allow separation of the alleles. One severe child is heterozygous for a common 392PvuII polymorphism and both of her alleles have been subcloned. We have determined that the moderately affected sons are heterozygous for an intronic polymorphism in IVS12 of COL1A2.

In addition, we wish to investigate whether the variable phenotype is associated with differences in the level of expression of mutant and normal α1(I) alleles. For this purpose, we probed total RNA with allele-specific antisense oligonucleotides spanning the region of the mutation. We demonstrated that in fibroblasts the ratio between normal and mutated transcripts does not correlate with phenotype.

We are also investigating a family in which a mother and child with an α2(I) gly 247 → cys mutation have type I and type III OI, respectively. We have identified a dimer band of α2(I) chains, which leads us to speculate that this family may have a mutation which affects chain incorporation, as well as the structural defect, because a standard type I collagen triple helix contains only 1 copy of the α2 chain. We have cloned both α2(I) alleles of the child and the C-terminal extension of the α1(I) chain to investigate this possibility.

9) In a patient affected by lethal OI type II, we have identified a gly421→asp substitution which generates an unusual electrophoretic mobility in the α2(I) chain. Electrophoretically, the proband's collagen has a broad α1(I) chain, a normal α2(I) and an α2(I) migrating half way between α1(I) and α2(I). The electrophoretic abnormality persists in the presence of α,α'-dipyridyl, suggesting an insertion or duplication in α2(I). The mutation was localized by CNBr digestion. Sequencing revealed a G→A transition at nt 1671 in one allele. This is the first case, in the α2(I) chain, of a single amino acid substitution resulting in a structural alteration of the collagen independent of excess post-translational modification. This behavior suggest the presence of a kink in the mutated α2(I) chain.

10) We have continued to screen the type I collagen of OI patients both as a genetic service to families and to identify cases of particular research interest. About 20 new skin samples on individuals with connective tissue disorders and their families were received this year. Of particular interest have been the fibroblast

collagens of patients from whom we are also studying the cultured osteoblasts. These fibroblasts are of interest not only for determination of the underlying mutation but also for comparison of expression and secretory patterns between different cell types. One CVS samples was screened this year and was determined to be normal.

11) We are continuing our studies of Ehlers-Danlos Syndrome (EDS), a connective tissue disorder whose symptoms overlap those of OI but do not include brittle bones. We are currently investigating nine cases of EDS.

In four of these cases, we find the same delay of electrophoretic migration of type I collagen chains and specific CNBr peptides, as is routinely found in OI. In three of these cases, unmodified collagen chains also have abnormal gel electrophoretic migration, suggesting the possibility of substitutions for non-glycine residues.

In one EDS case, the type I collagen chains are more rapidly migrating than normal, suggesting generally decreased modification. We are collaborating with Dr. Heather Yeowell at Duke University. She has demonstrated decreased activity of lysyl hydroxylase in the fibroblasts of these patients. We have now supplied her with cultured cells from this patient and are awaiting PCR analysis of the lysyl hydroxylase of this patient with EDS type VI.

We are investigating 4 cases of presumptive type IV EDS, by clinical criteria. We have utilized the technique of delayed reduction of type III collagen on SDS-Urea-PAGE to demonstrate electrophoretic abnormalities in two of these cases. In one case there is a greater than 80% reduction in the quantity of type III collagen, the remaining collagen III has normal electrophoretic migration. In the second case, there is the unusual situation that the amount of type III collagen is essentially normal, but it has more rapid electrophoretic mobility. We have begun to analyze the mRNA of these patients by PCR, looking for evidence of exon splicing defects especially in the case with more rapid electrophoretic migration.

12) We have been engaged in a collaborative project with our consulting orthopedic surgeon, Dr. C.M. Reing, and the Smith and Nephew Richards, Co. for the development and testing of alternative rodding materials for children with osteogenesis imperfecta. The Richards Co. has now been able to formulate resorbable rods with the desired breakdown time and we are ready to proceed with Phase II of the project.

13) We have continued our clinical protocol for the Growth Hormone Treatment Trial in Osteogenesis Imperfecta. At the completion of the study, we will have treated 26 children, including 9 type III OI and 17 type IV OI, for at least one year. From the first 20 children treated, 9 children responded with a sustained increase in linear growth rate of at least 50% over their baseline rate. The responder group was composed almost entirely (8/9) of children with type IV OI. Two-thirds of the type IV children were responsive to growth hormone treatment. We did not detect a significant change in bone density. The total patient group showed significant increases in IGF-I and IGFBP-3 by six months of treatment, continuing to 12 and 18 months. By one year of treatment, the IGF-I of the responder group exceeded that of the non-responders. For the total treatment group, we also detected a positive response of two markers of bone matrix synthesis, osteocalcin and type I collagen peptide. In the interim analysis of bone histomorphometric parameters, the responders experience a significant increase in bone volume/total volume, mineralized bone volume/total volume and bone formation rate/total volume. The non-responders have a significant decrease in trabecular thickness, osteoid volume/bone volume and osteoid maturation time.

14) We continued our clinical trial, in collaboration with Dr. Lynn Gerber of the Clinical Center Rehabilitation Medicine Department, of the effects of active rehabilitation and lower limb bracing on children with moderate and severe OI. The cross-over study of weaning OI children from braces was completed. The parameters evaluated include muscle strength, fracture rate and activity. There was more

decline in muscle strength in the unbraced period. Levels of strenuous activity were greater during the braced period. There was no statistically significant difference in fracture rate during braced and unbraced phases of this trial. Several of the OI children were able to be weaned without increase in fracture number.

15) We have continued our interest in the neurological aspects of OI, in collaboration with Dr. Lawrence Charnas. The clinically significant neurological finding is basilar invagination and consequent brainstem compression. This secondary feature of OI and the potential seriousness of its consequences, was unappreciated prior to this collaboration. Our interests include the delineation of the natural history of BI and determining the optimum nature and timing of the surgical response. Our patient population currently includes 15 children with documented basilar invagination. We have identified three physical parameters which correlate with the presence of BI in our population. Head circumference at or greater than the 98% has been 25% specific and 47% sensitive for predicting BI. An increase in head circumference by at least two standard deviations over a two to four year period of time has proven to be 100% specific and 60% sensitive in our group. A majority (12/15) of our patients with BI have a pronounced "occipital shelf". Anatomic BI may progress to syrinx formation or symptomatic obstruction in the teen years.

16) We have continued our collaboration with Dr. A. Yergey for the analysis of calcium kinetics in children with OI. At the conclusion of the growth hormone trial, 16 of the 26 children will have had calcium kinetics before and after treatment. Preliminary results of stable isotopic calcium metabolism/distribution studies in Types III and IV OI, showed an increased rate of calcium accretion to bone after treatment (type III OI, 94 ± 29 mg/kg/d pretreatment vs 142 ± 75 mg/kg/d post treatment, $p \geq .1$ and type IV OI, 64 ± 14 mg/kg/d pretreatment vs 91 ± 19 mg/kg/d post treatment, $p = 0.012$). While suggestive of important consequences, the numbers will be re-analyzed including the 4 patients from the final treatment group.

Significance to Biomedical Research and Program of the Institute:

This project attempts to elucidate the molecular biology of several genetic diseases of connective tissue at the gene, cell and tissue levels and to apply these findings to the treatment of the disorder.

We are interested in determining the relationship between the type and location of mutations in types I and III collagen and the pathophysiology of OI and EDS. These studies have broad implications for our understanding of skeletal biology and the interactions of the components of matrix. The study of OI mutations has led us to a Regional Model which suggests that some regions of the chains have an absolutely crucial role in higher order structures; we can now do binding experiments to try to determine the interactions for which the lethal regions are crucial. In addition, as dominant disorders of structural proteins, these disorders are excellent model systems in which to study the general genetic phenomena of variability of expression and parental mosaicism.

Because of the role of collagen as a structural, rather than an enzymatic protein, future gene therapy of these disorders will require that the defective allele be selectively turned off. We have been using antisense hammerhead ribozymes *in vitro* to demonstrate allele-specificity and determine the parameters for efficiency of cleavage. Further development of this novel form of gene therapy may include modifications to enhance selectivity, vector mediated stable transduction, and targeting of osteoblasts.

Development of a mouse model for moderately severe OI would allow testing of the new treatment modality in marrow stem cells. We are using site-directed mutagenesis and homologous recombination in embryonic stem cells to introduce a mutation into the mouse genome. The timing of expression of the mutant allele has been placed under the control of the cre-lox recombination system by the insertion of a lox-stop-lox transcription termination cassette.

We have recently turned our attention to the bony matrix in OI by initiating studies of cultured OI osteoblasts. The primary defect of collagen has secondary consequences for the non-collagenous proteins of bone and for the interactions of osteoblasts with matrix, resulting in a globally abnormal matrix. Studies of the structure and control of expression of osteoblast products in OI verses normal cells will enable us to understand how the collagen mutations are translated into a defective skeletal phenotype. This insight into osteoblast and matrix biology has broad implications for the management of other disorders of matrix or mineralization.

Our clinical protocols are conducted for the most part with the same patients whose collagen is studied in the laboratory, enabling us to correlate the patients' clinical response, bony matrix characteristics and primary collagen defect. Our interest in growth characteristics of abnormal matrix is reflected in our growth hormone treatment trial, testing the effect of hormone on linear growth and on matrix quality, its mineralization, cellular and trabecular structure. We plan to enlarge our growth studies to examine the plateau phase of growth in OI (ages 1-4 yrs) and the effects of puberty. We continue to lead a multidisciplinary effort in aggressive physical rehabilitation of children with OI to maximize functional level.

Proposed Course:

1) We plan to continue our development of selective antisense suppression of mutant collagen as an approach to therapeutic intervention in OI. Given the success of our *in vitro* experiments, we are now prepared to do studies with ribozymes in cultured cells from OI patients. We plan to target a number of mutations which generate a novel RZ cleavage site in both $\alpha1(I)$ and $\alpha2(I)$. Ribozymes with complementary arms designed to bind specifically to the sequences surrounding the OI cleavage site will be cloned into a pCI-neo mammalian expression vector. Ribozymes containing a mutation which inactivates the cleavage function will also be constructed, to control for antisense effects, as well as ribozymes with missense binding arms, to control for non-specific effects.

Ribozyme expression vectors will be introduced into cells by cationic liposome-mediated transfection. Transfected cells will be selected with G-418. The effectiveness and specificity of these ribozymes will be determined by monitoring the levels of mutant and normal type I collagen mRNA and protein. The levels of mutant and normal mRNA will be monitored by quantitative PCR; the mutations have been selected for study because they also change a restriction enzyme cleavage site. Normal and mutant protein will be monitored by proline labelling, electrophoretic separation of the chains and quantitation by densitometry. Toxic effects on the cells will be monitored by cell growth rates, level of protein synthesis and cell morphology. We also plan to study the effect of co-localization of ribozymes and type I collagen mRNA by adding type I collagen 3'-UTR sequences to ribozymes. Both full-length and partial 3'-UTR sequences will be tested for their ability to co-localize with type I collagen mRNA.

Ribozymes could be produced with high intracellular expression and with stable gene delivery by the use of a retroviral construct. There are currently available two such vectors, which use pol III tRNA promotors and termination signals to achieve high transcription levels and expression in a wide range of tissues. Since retroviral vectors are still preferred for stable delivery of genes to hematopoietic cells, these constructs would be suitable for transfer to stem cells. We would plan to test the *in vivo* effectiveness of such constructs in tissue culture and, eventually, in an appropriately engineered transgenic mouse.

2) We will continue to develop a murine model for non-lethal OI in collaboration with Dr. Heiner Westphal's lab. The primary purpose of such a murine model would be as a testing subject for selective antisense therapy. Additional purposes for such a model would be to provide a continuous supply of osteoblasts for study and for the testing of potential drug regimens. We will first mate our chimeras with wild type mice to check for the presence of the mutated allele in the germ line. In the first generation, we

expect a mouse carrying a null COL1A1 allele because of the presence of the lox-stop-lox cassette. This type of null allele has been associated with the mild type I form of OI. We will perform histological, biochemical and molecular studies to characterize the phenotype of the F1 mice. The expression of the mutant allele will be achieved in the second generation by mating the F1 mice with mice expressing cre recombinase. We plan to use a cre mouse in which the cre recombinase gene is under the control of the adenovirus EIIa promotor, thus targeting the expression of cre to the early mouse embryo. We expect an F2 generation with a phenotype similar to the severe non-lethal type IV OI. We will perform histological, biochemical and molecular analyses to characterize the F2 mice. Finally, we will develop a gene therapy approach using hammerhead ribozymes to selectively suppress the mutated allele and transform the severe OI phenotype into the mild form of the disease.

3) We will expand our investigations of the cultured osteoblasts from patients with OI. The goal of these investigations is to correlate the molecular defects of collagen with biochemical and molecular abnormalities of osteoblasts in order to understand how the mutations cause a range of phenotypic abnormalities.

First, we will follow up on our observation that some OI osteoblast collagens display different gel electrophoretic migration than fibroblast collagen from the same patient while other OI fibroblasts and osteoblasts synthesize collagen with the same electrophoretic abnormality. We are completing the identification of the causative collagen mutations in each of these OI cases. This will allow us to determine if there is a correlation of mutation location, in a particular chain, for example, and the behavior of osteoblast collagen. Our current hypothesis is that the osteoblast is more "tolerant" of mutant collagen than the fibroblast, resulting in less intracellular degradation and more secretion of the mutant helices. To test this hypothesis, we will compare degradation of the mutant chain in fibroblasts and ostoblasts by pulse chase experiments in an OI case with a cysteine substitution.

We also plan to investigate the non-collagenous products of the OI osteoblasts and relate their quantity and structure to the primary collagen abnormality. We plan to use Northern blots, Western blots, and antibody precipitations. We will study the synthesis, secretion and gel electrophoretic mobility of the proteoglycans biglycan and decorin, the bone specific proteins osteocalcin and BSP, and other bone proteins such as osteopontin and osteonectin.

We are also interested in the ability of the OI osteoblast to respond properly to exogenous growth factors. We plan to continue our studies of the differential response of OI and control OB to TGF-β. For the case with decreased response of collagen and proteoglycans, we will also probe for osteocalcin, osteonectin and bone sialoprotein. We plan to complete similar stimulations in several other cases of type IV OI, to determine whether this is a general feature of this type OI or related to specific mutations. At the protein level, we plan to follow the effect on production of type I collagen protein and select non-collagenous proteins. For type I collagen, we will also determine the effect on the electrophoretic migration of OB collagen. In those cases with generally decreased osteoblast responsiveness, we will examine TGF-β receptors and cell-matrix interactions of the osteoblasts.

We are also interested in the response of OI OB to IGF-I and GH, especially to compare osteoblasts from children who were clinically responsive or non-responsive to GH treatment. Furthermore, we plan to study the ability of OI OB to synthesize and secrete growth factors. Using conditioned media from our cultured OI osteoblasts, we can do ligand blots with labelled IGF to test for the presence of IGF-I binding proteins.

4) We will continue our molecular investigations of germ line mosaicism, focusing on families in which clinically normal parents have more than one child with a dominant form of OI. We will define the causative mutations in the affected offspring, then use WBC genomic DNA to determine which parent is the carrier. We will then explore expression of the mutant allele in a variety of tissues of the carrier. In our family with an $\alpha1(I)$ deletion/insertion, we have identified the paternal grandmother as the mosaic

carrier. When she died recently of an unrelated stroke, the family made a complete autopsy available. This will provide very valuable and indeed, unique, scientific data. We will be able to study the expression of mosaicism in virtually all organs and tissues using *in situ* PCR of paraffin imbedded sections. Because germ line mosaicism appears to be present in up to 10% of clinically normal couples who have had a single OI child, development of timely prenatal screening is an important clinical as well as genetic problem.

5) We will continue to delineate the molecular defects in the type I collagen of osteogenesis imperfecta patients using a combination of the RNA hybrid techniques developed in this lab and SSCP. Delineation of additional natural mutations is valuable because (1) this will allow us to further define the boundaries of the crucial and non-crucial regions in our regional model of OI pathophysiology; (2) knowledge of point mutations is essential information for studies on variable expression, mosaicism, antisense suppression, osteoblast metabolism and correlations with clinical data. We now have frozen fibroblasts from 25 cases in which we know the CNBr peptide pattern and from 65 additional cases in which electrophoretic abnormalities of the collagen chains have been demonstrated. Cases in which osteoblast samples are available for correlation or the family genetic pattern is particularly interesting will also be prioritized.

We will also continue our collagen protein screening on OI fibroblasts and CVS as a genetic service to families and as a source of new cases.

6) We plan to use the mutant type I collagens which we will purify for binding studies to investigate the possible functions of the "crucial" regions of the collagen chains identified in the regional model. One possible role for crucial regions would be to engage in collagenous or non-collagenous interactions. We have a collaborative agreement with Dr. Robert Spiro of La Jolla for the first of these binding investigations. We will provide Dr. Spiro with mutant type I collagen which he will investigate in his gel retardation assay for binding to decorin.

To investigate collagen interactions with other non-collagenous proteins, the target collagen, in either intact helical form or in heat denatured form, would be used to coat polystyrene microtiter wells. Collagen-coated wells would be exposed to a range of concentrations of normal type I, III and V collagen, osteonectin, osteocalcin (bone gla protein), osteopontin (BSP I), BSP II, and decorin (PG II). Dependence of binding of BSPs on calcium would also be investigated. Binding data would be subjected to Scatchard analysis.

7) We plan to pursue our studies on the molecular basis of variability of expression, concentrating on the $\alpha 1(I)$ gly 352 → ser mutation. We will consider collagenous as well as non-collagenous effects. For collagen, we will consider both structure and expression. We will complete the sequencing of the $\alpha 2(I)$ cDNA of the genetically relevant individuals to look for secondary mutations. We also have frozen cultured osteoblasts from two of the affected individuals. We will obtain mRNA from cultured osteoblasts and compare relative levels of expression of the mRNAs and secretory rates of mutant chain from the cells. The osteoblasts will also provide an opportunity to examine non-collagenous mRNAs and proteins. "Genetic background" may affect OI phenotype in a variety of ways. Some of these effects may be regulatory and difficult to investigate. However, secondary alterations in the amounts of interacting matrix proteins are reasonable and accessible candidates.

We are also studying variability of expression in a mother and her son with a cysteine substitution in $\alpha 2(I)$. We will complete the sequencing of both $\alpha 2(I)$ alleles in the child as well the C-terminal extension of $\alpha 1(I)$. If this does not yield any differences, we will determine the levels of the various chains of types V and III collagen, the minor collagens of skin and bone.

8) For the cases of Ehlers-Danlos Syndrome with evidence of structural abnormality in type I collagen but without clinical evidence of bone disorder, our goal is to identify the associated mutations at the molecular

level.

For the four cases whose electrophoretic abnormality persists in unmodified chains, there is the possibility that the mutations may be amino acid substitutions in the X or Y positions of the chains involving cysteine, proline or lysine residues. Standard protein techniques and analysis of PCR products by hybrid analysis or denaturing gels will be utilized to localize the mutations prior to sequencing.

We will continue our collaboration with Dr. Yeowell at Duke University to investigate the molecular defect(s) of lysyl hydroxylase in the fibroblasts of our patient with type VI EDS.

9) We will continue to investigate the cases of Ehlers-Danlos IV with reduced amounts of type III collagen. If the mutant type III collagen is incorporated into triple helix, we may be able to further localize the abnormality by CNBr peptides. Type III collagen may be separated from the much larger amounts of type I collagen produced by these cells using selective salt precipitation. We will then pursue the abnormality by PCR amplification of the appropriate region of type III collagen mRNA. We have prepared amplification primers along the chain and have also subcloned overlapping segments of type III collagen cDNA into pGEM vectors for the production of riboprobe for hybrid analysis. If the mutant type III collagen protein is not incorporated into the triple helix, it will be necessary to investigate the entire type III cDNA by PCR amplification and hybrid analysis.

10) We will be continuing our clinical protocol consisting of a treatment trial of growth hormone in OI. We have enrolled our final 6 patients and will conclude one year of treatment in all participants by the end of 1996. We will then be able to compare preliminary endocrine evaluations, and pre- and post-treatment bone density, bone morphometric properties, matrix peptides and growth factors in the group of OI children whose bone had a positive response to hormonal stimulation and in the non-responder group. Our goals are (a) to be able to predict which OI children will benefit from growth stimulation, and (2) to determine whether growth hormone exposure has a beneficial effect on bone density or bone morphometric properties apart from a stimulatory effect on linear growth.

11) We are planning to expand our investigations on growth in OI by submitting a protocol dealing with the plateau phase of OI growth (age 1-4 yrs). We have made the observation that growth of types III and IV OI children plateaus between ages 1-4 years. For the type IV children, it then resumes, often at a normal rate. To our knowledge, this time-limited phase of minimal growth has been described only in OI. We are planning to submit a protocol for sequential endocrine evaluation of this age range. The clinical importance of this study is that elimination of the plateau phase would eliminate extreme short stature in type IV OI.

12) Continued study of the neurological aspects of OI will be a major aspect of a protocol which will be submitted. OI children over 5 years of age will have a CT scan every 2 years to rule out the development of basilar invagination. For those children in whom BI is detected, follow-up studies will consist of a yearly MRI to obtain better soft tissue images for the detection of brain stem compression. The children will also receive semi-annual evaluation by a neurologist who has expertise in connective tissue disorders. These studies will allow us to follow the development of BI as well as the frequency and rate of progression. This information, in turn, will allow us to refine our treatment plan for this serious secondary feature of OI.

13) We are continuing our collaborative interest in the physical rehabilitation of children with OI in collaboration with Dr. Gerber, Chief, PM & R Department, NIH CC. We are now recruiting patients for a prospective randomized trial of bracing in OI children under age 3 years who have not yet attained ambulation.

For children ages 5-15 years, our clinical protocol will enable us to document the effects of an aggressive rehabilitation approach to OI on the long term maintenance of ambulation and on the secondary features of OI, including the spine, pulmonary and cardiac function and neurological aspects.

14) We will continue our collaborative study for the development of alternative rodding materials. In the area of resorbable rods, we will use the rodding material which has been formulated by Richards and Co. to stabilize femur fractures in rabbits. Resorbable rods will allow OI bone to gradually resume full weight bearing and avoid a surgery for removal.

Animal Protocols:

91-047	Marini	New Materials for Intramedullary Rods

Clinical protocols:

92-CH-34	Marini	Treatment Trial of Growth Hormone in Osteogenesis Imperfecta
89-CC-116	Gerber	Study of the Effects of Bracing on Ambulation, Bone, Density and Functional Activity in Children with OI

Publications:

Byers PH, Marini JC, Gerber L. Frontiers in rehabilitation medicine: Osteogenesis Imperfecta, Overview of a Conference. Conn Tissue Res. (1995) 31:253-255.

Charnas LR, Marini JC. Neurologic profile in osteogenesis imperfecta. Conn Tissue Res. (1995) 31:S23-S26.

Grassi G, Marini JC. Ribozymes - Future tools for treatment of dominant diseases. in press; Annals of Medicine.

Marini JC, Bordenick S, Chrousos GP. Endocrine aspects of growth deficiency in OI. Conn Tissue Res. (1995) 31:S55-S57.

Wang Q, Forlino A, Marini JC. Alternative splicing of COL1A1 mRNA leads to a partial null allele and two in-frame forms with structural defects in non-lethal Osteogenesis Imperfecta. in press; J. Biol. Chem

Wang Q, Marini JC. Antisense oligonucleotides selectively suppress expression of the mutant α2(I) collagen allele in OI type IV fibroblasts: A molecular approach to therapeutics of dominant negative disorders. J. Clin. Invest. (1996) 97:44-454.

Yergey AL, Vieira NE, Abrams SA, Marini JC, Goans RE. Use of stable isotopic tracers in studies of whole body calcium metabolism. Conn Tiss Res. (1995) 31:291-293.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00910-17 HDB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Uteroglobin, Phospholipase A_2, and Osteopontin Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Anil B. Mukherjee, M.D., Ph.D.	Head	HGB, NICHD
Others:	Gopal C. Kundu, Ph.D.	IRTA Fellow	HDB, NICHD
	Gary Zhang, M.D., Ph.D.	IRTA Fellow	HDB, NICHD
	Peter Yuan, Ph.D.	Visiting Fellow	HDB, NICHD

COOPERATING UNITS *(if any)*

Georgetown University, McGill University, Harvard Medical School and Beth Israel Hospital, Naval Research Laboratory, Washington Hospital Center/Medlantic Research Foundation, University of Florence, Italy.

LAB/BRANCH

Heritable Disorders Branch

SECTION

Developmental Genetics

INSTITUTE AND LOCATION

NICHD, Bethesda, Maryland 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	4.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have:(a) uncovered a novel physiological role of uteroglobin (UG) in preventing severe glomerular inflammatory disease by gene targeting in mice; (b) characterized the entire human UG gene and discovered a polymorphism in Best's disease patients and their family members; (c) discovered that tumor cells, originating from organs which normally express UG gene, do not produce UG and induced overexpression of UG gene in these cells dramatically alters their tumorigenic phenotype in that they fail to have anchorage-independent growth on soft agar (a marker for transformation and tumorigenesis) and have low tumorigenic potential in nude mice, raising the possibility that UG may have tumor-suppressor role; (d) delineated the crystallographic structure of recombinant human UG (hUG) at 1.8 Å resolution; (e) resolved the solution structure of recombinant hUG by multidimensional NMR and CD; the results suggest that this protein is nearly identical to rabbit UG; (f) characterized a novel high-affinity cell surface receptor of UG via which UG regulates ECM-invasion in several cell types. (g) established that group I PLA_2 via its high-affinity receptor induces ECM-invasion in normal cells. Interestingly, these receptors are overexpressed in several tumor cells; (h) discovered that a mouse model which expresses osteopontin (OPN) antisense mRNA at high level in the mammary gland manifests abnormality in mammary gland development and differentiation; (i) designed and constructed OPN targeting vector transfection of which in ES cells resulted in heterozygotic cells for targeted disruption of OPN gene; these cells will be used to generate OPN knockout mice; (j) demonstrated by using an *in vitro* assay system that cells lacking proto-oncogene *c-fos* fail to express OPN gene; since OPN is required for normal osteoclast attachment to the osteoid, a process essential for bone remodeling, our results, at least in part, explains why *c-fos*$^{-}/_{-}$ mice would develop osteopetrosis and (k) established that high level expression of OPN gene in human coronary artery smooth muscle cells play a critical role in cellular migration, ECM-invasion and proliferation, essential for coronary restenosis after angioplasty, a frequently used procedure for the treatment of coronary atherosclerotic disease.

Project Description:

I. Objectives:

Diseases caused by over-activation or suppression of immune responses are important causes of morbidity and mortality. Although the familial nature of several primary inflammatory diseases (eg. atopic asthma, rheumatoid and juvenile rheumatoid arthritis, autoimmune uveitis and psoriasis) is well recognized, the molecular and genetic mechanisms of pathogenesis of these diseases are still not clearly understood. Investigations in the general area of cell biology, immunology, molecular biology and genetics of inflammation have led to the discovery of genes which appear to play important role(s) as biological modulators (i.e. activators and inhibitors) of inflammation. For several years, one of the objectives of our laboratory has been to investigate the structure-function relationships of several genes and their products which directly or indirectly modulate immune and inflammatory processes. These include genes encoding phospholipases A_2 (PLA_2; E.C.3.1.1.4), the uteroglobin (UG)/Clara cell 10kDa (cc10kDa) family of proteins and osteopontin (OP). Within the last year we discovered a high-affinity cell surface receptor for human uteroglobin. Thus, our overall objectives also include: (a) cloning and characterization of the cDNA coding for this receptor and (b) delineation of the mechanism(s) of signal transduction via this receptor pathway in health and in disease.

A thorough knowledge of the mechanism(s) of action and regulation of these genes and their products is expected to improve our understanding of the molecular and genetic basis of some of the above mentioned diseases that affect a significant portion of our population and facilitate the development of rational and novel therapeutic approaches. To achieve these objectives, we have focused on investigations, the methods of approach and results of which are summarized below:

METHODS EMPLOYED:

State of the art methodologies of recombinant DNA technology, protein chemistry, cell biology and immunology are used. More specifically, we use all techniques related to gene cloning and sequencing, PCR, RT-PCR, *in situ* hybridization, Northern, Southern and Western blotting, generation of transgenic mouse model and gene targeting by homologous recombination ("knock out"), expression of recombinant proteins in *E. coli*, site-directed mutagenesis, radioimmunoassay, ELISA, tissue culture, cell invasion assay, immunohistochemistry and immunofluorescence. In collaborative studies, we applied X-ray crystallography and multidimensional Nuclear Magnetic Resonance (NMR) for structure-function analysis of recombinant proteins. Advanced enzymological and kinetic analyses are also used.

PROGRESS REPORT:

During the period since our last annual report we have accomplished the following:

a. **Targeted disruption of the uteroglobin (cc10kDa) gene in mice by homologous recombination:** While many biological effects of uteroglobin have been described, the physiological role(s) of this protein remains obscure. In order to determine the physiological role of this protein, we undertook the targeted disruption of UG gene in mice by homologous recombination. The mouse gene, like its human counterpart, consists of three exons and one intron. By using standard techniques, we have successfully disrupted this gene in ES cells and isolated and characterized three different ES clones. When these cells were injected in the blastocyst and transferred them to surrogate mothers live chimeric progeny were born. The founders (chimeras) were further mated with C57BL/6 and obtained F1 mice, a number of which tested positive (heterozygote) for carrying the null mutation in UG gene (i.e. UG^{+}/$_{-}$). The F1 mice carrying the mutation attested that successful germline transmission of the mutation has been achieved. Mating of the heterozygotes (F1 X F1) obtained from breeding each of the two independent founders generated mice homozygous for UG gene disruption (i.e. UG^{-}/$_{-}$). UG -/- mice, developing severe renal glomerular inflammatory disease, become cachectic and die within 4-5 weeks after birth. The mechanism of pathogenesis of this disease is now being delineated.

b. **Cloned and characterized the human UG gene and identified a polymorphism of this gene in patients with Best's macular degeneration and their family members:**
The cDNA encoding hUG and the 5' promoter region of the UG gene have been characterized previously. We now found that the structure of the entire hUG gene is virtually identical to those of the rabbit, rat and mouse. It is localized on human chromosome 11q12.8-13.1, a region in which several important candidate disease genes have been mapped by linkage analyses. Our data indicate that candidate genes for atopic (allergic) asthma and Best's vitelliform macular dystrophy are in closest proximity to the hUG gene. In order to determine whether hUG gene mutation may be involved in the pathogenesis of these diseases, we studied two isolated groups of patients, each afflicted with either atopy or Best's disease respectively. We detected a single base-pair change in the hUG gene in Best's disease patients and normal controls but no such change was detected in atopy patients. This alteration in hUG gene-sequence in Best disease family appears to be a polymorphism. While the results of our investigation did not uncover mutations in hUG gene which could be causally related to the pathogenesis of either of these diseases, its conservation throughout vertebrate phyla implies that this gene is of physiological importance. Moreover, the close proximity of this gene to several candidate disease genes makes it an important chromosomal marker in cloning and characterization of those genes.

c. **Tumor suppressor effect of UG gene overexpression in tumor cell lines:**
Recently, we have reported the characterization of a putative cell surface receptor for human uteroglobin (hUG). Via this receptor-mediated pathway hUG appears to inhibit cellular invasion of extracellular matrix. It has been reported that many epithelial tumors (eg.lung & uterus), fail to express hUG while their normal counterparts do so at a high level. In addition, there are reports suggesting that a tumor suppressor gene may be localized in human chromosome 11q^{13}, a region in which hUG gene colocalizes. Thus, we sought to determine the effect(s) of constitutive overexpression of hUG gene on the phenotype of these cells. We transfected several tumor cell lines derived from adenocarcinomas of the lung (HTB-174), uterus (HTB-111), prostate (HTB-81) and breast (HTB-30). Constitutive overexpression of the hUG gene was achieved in these cells by transfecting them with a plasmid construct (pRc/RSV; *Invitrogen*) containing a full length hUG cDNA under the control of RSV promoter. Control cells were either non-transfected or transfected with the plasmid vector only (mock). We determined the expression of hUG mRNA and protein after neomycin selection of the clones by RT PCR and Western blotting respectively. Both transfected and control cells were tested for their rate of proliferation and their ability to grow on soft agar. We found that hUG-transfected cells expressed hUG mRNA as well as protein at a high level. The rate of proliferation of hUG-transfected cells were dramatically slower than the controls. Most significantly, while the control cells proliferated rapidly on soft agar, the hUG-transfected cells failed to grow although they were viable. The tumorigenicity test using nude mice are now being carried out. The striking phenotypic change we observed in hUG-transfected tumor cells and the dramatic reversal of their transformed phenotype raise the possibility that hUG gene may have tumor suppressor effect. Most interestingly, this effect

appears to be specific for endometrial adenocarcinoma cells but not of those originating from the lung, breast or prostate.

d. **Resolution of recombinant human UG structure by X-ray crystallography:** In light of the fact that human uteroglobin has recently been found to have a high-affinity binding on several cell types and that binding of hUG onto this receptor affects cellular invasion of extracellular matrix prompted us to determine the structure of this protein. Delineation of its structure will allow us to determine the region of hUG molecule involved in receptor-ligand interaction, knowledge of which is would facilitate our understanding of the signal transduction via this hUG receptor. This knowledge may facilitate development of hUG receptor agonists which may be clinically useful in preventing cellular migration and invasion in inflammatory as well as in neoplastic diseases. In collaboration with Drs. Ward and Pattabiraman, we have resolved the human uteroglobin structure at 2.1Å resolution of X-ray crystallography. The monoclinic crystals used for diffraction studies yielded lattice constants a=69.2Å, b=83.0Å, c=58.3Å and β=99.7°. These results suggest that the secondary structures of rabbit and human UG are extremely similar, if not identical. Attempts are now being made to obtain even finer resolution of the crystal structure of hUG.

e. **Solution structure of recombinant human uteroglobin resolved by multidimensional NMR:** Since hUG is a soluble protein, it is important to determine the conformation of this protein in solution in order to understand what regions (or domains) of this protein are exposed to the solvent and therefore, available for interaction with other molecules. We expressed both unlabeled as well as ^{15}N and $^{15}N/^{13}C$-labeled recombinant hUG in *E.coli*. These proteins were purified to homogeneity and NMR studies were performed in collaboration with Dr. Gaetano Barbato at the IRBM, Rome Italy. Preliminary results of both circular dichroism (CD) spectroscopy as well as NMR suggest that hUG is a highly helical protein in solution. The hUG molecule in solution was found to be extremely stable. The apparent T_m appears to be above 70°C and a complete recovery of the native folding was possible at pH 6.2. The results of CD spectroscopy and NMR are in good agreement as regards to its T_m and α-helical structure. A comparison of these data with those from rabbit UG indicates that these two molecules are nearly identical structurally. Since our preliminary results of site-directed mutagenesis studies indicae that mutation of one lysine residue in α-helix 3 abolishes PLA_2 inhibitory activity of hUG, studies in progress are attempting to determine what structural constraints are brought about as a result of such mutations.

f. **Recombinant human UG suppresses cellular invasiveness via a novel class of high affinity cell surface binding site:** The mechanism(s) that regulates invasion of trophoblasts through the uterine epithelium during embryo implantation and nidation in hemochorial placental mammals is poorly understood. While limited trophoblast invasion is essential for the establishment of normal pregnancy, dysregulation of this process may contribute to the pathogenesis of choriocarcinoma, a highly invasive and lethal form of cancer arising from the trophoblasts. We have previously demonstrated that rabbit uteroglobin (UG), a cytokine-like, antiinflammatory protein, produced by the endometrial epithelium during pregnancy, has a potent antichemotactic effect on neutrophils and monocytes *in vitro*. Here, we report that recombinant human UG (hUG) dramatically suppresses invasion of human trophoblasts and NIH 3T3 cells through an artificial basement membrane (Matrigel) *in vitro*, but has no effect on that of human choriocarcinoma cells. We identified, for the first time, a novel, high-affinity, high molecular weight (_190kDa), non-glycosylated hUG-binding protein, readily detectable on human trophoblasts and NIH 3T3 cells but totally lacking on choriocarcinoma cells. Taken together, these results raise the possibility that (i) hUG plays a critical role in regulating cellular invasiveness, at least in part, via its novel cell surface binding site, and (ii) some of the numerous biological activities of proteins of the UG family, reported so far, may be mediated via this novel binding site.

g. **Evidence that porcine pancreatic phospholipase A_2 via its high-affinity receptor stimulates extracellular matrix invasion by normal and cancer cells:** Cellular migration and extracellular matrix (ECM)-invasion are some of the critical steps in embryonic implantation, inflammation, wound healing and cancer metastasis. Extracellular phospholipases A_2 (PLA_2s), belonging to either group I (PLA_2-I) or group II (PLA_2-II), play critical role(s) in the generation of proinflammatory lipid mediators (eg.prostaglandins, leukotrienes, platelet activating factor etc.). Recent reports indicate that PLA_2-I, in addition to its digestive function, have receptor-mediated effects. For instance, PLA_2-I, via its receptor can induce cell proliferation and airway as well as vascular smooth muscle contraction. Here, we report that porcine pancreatic PLA_2, in a dose-dependent manner, stimulates dramatic invasion of an artificial ECM (Matrigel^{TM}) by NIH 3T3, mouse fibrosarcoma and sarcoma cells *in vitro* but it has no such effect on lymphoma and mastocytoma cells. That this is a receptor-mediated process is strongly suggested by the following findings: (a) while NIH 3T3, mouse fibrosarcoma and sarcoma cells, which respond to PLA_2-I stimulation, express high affinity PLA_2-I receptor mRNA and protein, this receptor expression is not detectable in non-responder lymphoma and mastocytoma cells, (b) there is a close correlation between the K_d values for ^{125}I-PLA_2-I binding to its receptor and the ED_{50}s for PLA_2-I-induced ECM-invasion, (c) catalytically inactivated PLA_2-I is as effective as active enzyme in stimulating invasion, (d) transfection of those cells which express PLA_2-I receptor with its antisense S-oligonucleotide, abrogates their ability to invade the ECM and (e) treatment of the same cells with PLA_2-I receptor antibody also yields similar results. To our knowledge, this is the first report of this novel receptor-mediated effect (ie. ECM-invasion) of PLA_2-I in several normal and malignant cell types of the fibroblastic but not of malignant myelogenous. The fact that this receptor is expressed by different cell types *in vitro* and several organs *in vivo* raises the possibility that extracellular PLA_2s play important physiological as well as pathological roles via this receptor.

h. **Dramatic abnormality in mammary gland development and differentiation in mice carrying an antisense osteopontin (OPN) transgene:** In collaboration with Dr. Barid Mukherjee, McGill University, Canada, we have generated two independent lines of transgenic mice carrying antisense OPN cDNA as the transgene under the control of MMTV-LTR promoter. We have now generated substantial number of these mice. These mice develop severe abnormalities in mammary gland development and differentiation which correlates well with the copy number of the transgene. The mice expressing OPN antisense mRNA at a high level do not lactate as a result of mammary gland abnormality and the offspring die due to lack of milk. Since MMTV-LTR is known to target transgenes to the mammary gland and since OPN is expressed at a high level in this gland and detectable in milk, these results suggest that OPN gene expression is important for mammary gland development and differentiation. Ongoing studies focus on delineating the mechanism(s) of this abnormality and the role of OPN gene in preventing its pathogenesis.

i. **Disruption of the osteopontin gene in mice by homologous recombination:** We have successfully constructed the targeting vector containing the disrupted osteopontin (OPN) gene and transfected the ES cells which yielded several positive clones in which OPN gene has been clearly disrupted. These cells are now being injected in blastocysts to produce chimeras in order to generate the OPN-null mice.

j. **Osteopontin gene expression is dramatically reduced in protooncogene c-fos-deficient**

osteopetrotic mice: Disruption of proto-oncogene c-*fos* in mice (i.e. c-*fos*$^{-}/_{-}$), by homologous recombination causes osteopetrosis due to impaired bone resorption by osteoclasts. Osteopontin (OPN), a secreted, non-collagenous, glycosylated phosphoprotein, is essential for osteoclast adherence to bone matrix, a prerequisite for bone resorption. We found that cells derived from c-*fos*$^{-}/_{-}$ osteopetrotic mice synthesize a drastically low level of OPN, but its expression is inducible by epidermal growth factor (EGF) or 12-O-tetradecanoyl phorbol-13-acetate (TPA) treatment. We have previously demonstrated that c-*src* plays a critical regulatory role in OPN gene expression, raising the possibility that similar mechanisms (lack of OPN) may underlie the pathogenesis of osteopetrosis in both c-*fos* and c-*src* deficient mice. Ongoing studies in *c-fos*-deficient mice as well as familial osteopetrotic patients may delineate the mechanism of this disease.

k. **Potential role of OPN gene expression in human coronary restenosis:** Angioplasty procedures are increasingly used to reestablish blood flow in blocked atherosclerotic coronary arteries. A serious complication of these procedures is reocclusion (restenosis) which occurs in 30-50% of patients. Migration of coronary artery smooth muscle cells (CASMCs) to the site of injury caused by angioplasty and subsequent proliferation are suggested mechanisms of reocclusion. Results of experiments, using cultured human CASMCs and atherectomy tissues from patients, identified osteopontin (OPN) and one of its receptors ($\alpha_v\beta_3$ integrin) as critical elements in CASMC-migration, extracellular matrix-invasion, and proliferation. These effects were abolished when OPN gene-expression or interaction with its receptor were blocked. These findings establish an essential role for OPN and $\alpha_v\beta_3$ integrin in coronary reocclusion.

COOPERATIVE RESEARCH AND DEVELOPMENT AGREEMENT (CRADA):

1. Knight, M. Collaborating Principal Investigator:Peptide Technologies Corp. Washington, D.C.

   Title of Project: Development of a radiometric assay for HIV-1 aspartic protease activity for screening potential inhibitors of this enzyme. Approved June 1, 1991.

INVENTION REPORTS:

A. Development of a Uteroglobin-deficient Mouse model for human hereditary renal glomerular disease;

B. Potential therapeutic application of OPN-antisense oligonucleotides in human coronary restenosis after angioplasty procedures.

Significance to Biomedical Research and to the Program Goals of the Institute:

Inflammation and tissue injury secondary to immunological activation characterize a number of relatively common human diseases such as bronchial asthma, rheumatoid and juvenile rheumatoid arthritis, autoimmune uveitis, and glomerulonephritis, to name a few. Together, these diseases affect a significantly large population of the US and the world. For instance, asthma is one of the most frequent causes of hospital admission for children and causes approximately 5,000 deaths per year in the US alone. In diseases like rheumatoid arthritis and asthma, inflammation has been suggested to play a primary pathogenic role. The following developments during the last year underscores the importance of basic research in finding answers to clinical problems:

(a) Uteroglobin-deficiency and hereditary glomerulopathies:
Our discovery that targeted disruption of uteroglobin gene in mice causes a glomerulonephritis, an

inflammatory disease, is not fully unexpected as uteroglobin is a potent antiinflammatory protein. The disease which develops in the UG -/- mice is virtually identical to hereditary glomerulonephritis in humans raising the possibility that the mechanism of pathogenesis and new avenues of therapy for this lethal disease may be found in the near future.

(b) Induced Overexpression of uteroglobin gene in endometrial adenocarcinoma cells alters their phenotype :
Our recent discovery that induced overexpression of UG gene in endometrial cells alters their transformed phenotype in that they fail to grow on soft agar is a very significant finding. These results coupled with our previous finding that human UG gene localizes in chromosome $11q^{12.8-13.1}$, a region in which a putative tumor suppressor gene may be located, raise the possibility that UG may have tumor suppressor activity.

(c) Potential role of osteopontin in coronary restenosis:
Results of our study with osteopontin (OPN) gene expression in coronary artery smooth muscle cells *in vitro* as well as in atherectomy samples from patients with severe coronary artery disease showed that OPN gene overexpression causes migration, ECM-invasion and proliferation of coronary smooth muscle cells,essential for restenosis after angioplasty procedures. In addition, the results of experiments in which smooth muscle cells were treated with OPN-antisense oligonucleotides showed that inhibition of OPN production can prevent cellular migration, ECM-invasion and proliferation raising the possibility that experiments in animal models can proceed to develop new therapeutic approaches for prevention of restenosis after angioplasty. Moreover, our more recent investigation on osteopontin gene is beginning to explain the etiology of some forms of osteopetrosis.

Our investigations on three functionally related genes (ie. UG, OPN and PLA_2), their involvement in the pathogenesis of genetic diseases of children and adults, have profound Public Health implications which make our research endeavor consonant with the mission of Heritable Disorders Branch and of the NICHD.

ANIMAL PROTOCOLS:

#92-037	Reagent Antibody Production
#93-007	Uteroglobin:(a) tissue-specific expression of the UG gene in developing rabbit and (b) possible therapeutic benefits of administration of artificial surfactant-recombinant human UG complex in the treatment of neonatal respiratory distress syndrome (RDS) in the rabbit.
#94-047	Determination of physiological role of uteroglobin by using UG-antisense transgenic mice.
#95-018	Determination of the physiological role of uteroglobin by gene targeting in mice.

PROPOSED COURSE:

Our future research plans include the following:

a. Study families with hereditary glomerular disease to determine whether hUG gene mutation is associated with this disease;

b. To further characterize UG "knockout" mice for other phenotypes and delineate whether therapy could be an alternative treatment for this disease;

d. To purify and characterize the human uteroglobin (hUG)-binding protein (putative receptor) and clone and characterize the cDNA and the gene coding for this protein;

e. To delineate the mechanism(s) of signal transduction via hUG receptor;

f. To further characterize the suppression of cellular invasion via hUG receptor-mediated pathway;

g. To further characterize the tumor-suppressor effects of UG in tumor cell lines;

h. To further characterize the relationship between c-Src and c-fos gene disruptions and suppression of OP gene expression with osteopetrosis;

i. To delineate the role of osteopontin gene in mammary gland development in OPN-antisense transgenic and OPN-"knockout" mice.

***LIST OF PUBLICATIONS*:**

Chackalaparampil, I., Peri, A., Nemir, M., McKee, M., Lin, P.H., Mukherjee, B.B. and **Mukherjee, A.B.**,: Cells *in vivo* and *in vitro* from osteopetrotic mice homozygous for *c-src* disruption show suppression of synthesis of osteopontin, a multifunctional extracellular matrix protein. *Oncogene* 12:1457-1467, 1995

Knight, M., Ito, Y., Chandrasekhar, B., Takahashi, K. and **Mukherjee, A.B.**: Purification of the human immunodeficiency virus-1 aspartyl protease substrate peptide.In: Amer. Chem. Soc. Symp. Series #593: *Modern Countercurrent Chromatography*. Conway, W.D. and Petroski, R.J. eds. Amer. Chem. Soc. Publ. 1995. Chapter 10: pp111-118.

Kundu, G.C. and **Mukherjee, A.B.** Evidence that porcine pancreatic phospholipase A_2 via its high-affinity receptor can stimulate extracellular matrix invasion by normal and cancer cells. *J. Biol. Chem.* **(in press).**

Kundu, G.C., Mantile, G., Miele, L., Cordella-Miele, E. and **Mukherjee, A.B.** Recombinant human uteroglobin suppresses cellular invasiveness via a novel class of high-affinity cell surface binding site. *Proc. natl. Acad. Sci. (USA)* 93:2915-2919 (1996).

Mathews, J.H., Pattabiraman, N., Ward, K.B., Mantile, G., Miele, L. and **Mukherjee, A.B.** Crystallization and characterization of the recombinant human Clara cell 10kDa protein. *Proteins: Structure, Function and Genetics*.20:191-196, (1995).

Mukherjee, B.B., Nemir, M., Beninati, S., Cordella-Miele, E., Singh, K., Chackaparampil, I., Shanmugam, V., DeVouge, M. and **Mukherjee, A.B.** Interaction of osteopontin with fibronectin and other extracellular matrix molecules. *Ann. N.Y. Acad. Sci.* 760:201-212 (1995).

Mukherjee, A.B., and Miele, L. Design of immunomodulatory peptides based on active site structures. In: *Structure-based Drug Design (William Williams and David Weiner eds)*. CRC Press, Inc. 1995, pp.237-261

Panda, D., Kundu, G.K., Lee, B.I., Peri, A., Chackalaprampil, I., Mukherjee, B.B., Li, X.D., Mukherjee, D.C., Seides, S., Rosenberg, J., Stark, K., and **Mukherjee, A.B.** Potential role of osteopontin and $\alpha_v\beta_3$ integrin in coronary restenosis *Nature* **(submitted).**

Peri, A., Dubin, N., Dhanireddy, R. and **Mukherjee, A.B.** Uteroglobin gene expression in the rabbit uterus throughout gestation and in the fetal lung: Relationship between uteroglobin and eicosanoid levels in the developing fetal lung. *J. Clin. Invest.* 96:343-353 (1995).

Shanmugam, V., Chackalaparampil, I., Kundu, G.C., **Mukherjee, A.B.** and Mukherjee, B.B. Sialic Acid Content Influences Specific Binding of Osteopontin to Temperature-sensitive Rat-1 (tsB77) Cell Surface *Biochemistry* **(in press)**

Su, L., Mukherjee, B.B. and **Mukherjee, A.B.** Expression of antisense osteopontin RNA inhibits tumor promoter-induced neoplastic transformation of mouse JB6 epidermal cells. *Oncogene* 10:2163-2169 (1995).

Xing, M., Miele, L. and **Mukherjee, A.B.** Arachidonic acid release from NIH 3T3 cells by group I phospholipase A_2: Involvement of a receptor-mediated mechanism. *J. Cell. Physiol.* 165:566-575 (1995).

Zhang, Z., Ward, J. M., Lee, E., Kundu, G.C., Yuan, P., De Mayo, F., Westphal, H. and **Mukherjee, A.B.** Mice homozygous for uteroglobin gene disruption (UG -/-) develop severe renal glomerular disease **(to be submitted).**

Zhang, Z., Zimonjic, D.B., Popescu, N.C., Wang, N., Gerhard, D., Stone, E., Arbour, N.C., de Vries, M.A., Scheffer, H., Garritsen, J., Collie', J., ten Kate, L. P. and **Mukherjee, A.B.** Human uterogobin gene: Its structure, subchromosomal localization, polymorphism and possible role in disease processes. *DNA & Cell Biol.* **(in press).**

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00912-17 HDB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene Regulation and Cellular Differentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Janice Y. Chou, Ph.D.	Head	HDB, NICHD
Others:	Kei-Jian Lei, Ph.D.	Visiting Scientist	HDB, NICHD
	Margaret Chamberlin, Ph.D.	IRTA Fellow	HDB, NICHD
	Hungwen Chen, Ph.D.	Visiting Fellow	HDB, NICHD
	Tsuneyuki Ubagai, Ph.D.	Visiting Fellow	HDB, NICHD
	Chun Zhao, Ph.D.	NRC Fellow	
	See attached		

COOPERATING UNITS *(if any)*

University of Florida (Dr. C-L Chen); Duke University (Dr. Y-T Chen); University of North Dakota (Dr. R Nordlie); NMH (Dr. SH Mudd); University of Virginia Children's Medical Center (WG Wilson); Institute of Child Health, London, UK (JV Leonard); Institute for Human Gene Therapy (JM Wilson).

LAB/BRANCH

Heritable Disorders Branch

SECTION

Cellular Differentiation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-1830

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.5	5.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Glycogen storage disease type 1 (GSD-1) is caused by a deficiency in microsomal glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis. We have generated *G6Pase*-deficient mice and shown that the knockout mice manifest essentially the same pathophysiology as human GSD-1a patients, indicating that the G6Pase$^{-/-}$ mouse is a valid animal model for the study of GSD-1a. To further understand the mode of G6Pase catalysis, we examined G6Pase in the liver and kidney of wild-type and *G6Pase* knockout mice and demonstrated that G6P transport and hydrolysis are performed by separate proteins which are tightly coupled. Currently, we are pursuing this *G6Pase*-deficient mouse model to develop gene therapy for GSD-1a.

Diagnosis of GSD-1a currently is established by demonstrating the lack of G6Pase activity in the patient's biopsied liver specimen. Our cloning of the *G6Pase* gene and identification of mutations within the gene that cause GSD-1a allow for the development of a DNA-based diagnostic method. We have characterized the *G6Pase* gene of 70 unrelated GSD-1a patients and uncovered sixteen disease-causing mutations. This DNA-based diagnosis now permits prenatal diagnosis among at-risk patients and serves as a data-base in screening and counseling patients clinically suspected of having this disease.

Methionine adenosyltransferase (MAT) is a key enzyme in transmethylation, transsulfuration, and the biosynthesis of polyamines. We have characterized the human *MAT1A* gene and shown that individuals deficient in hepatic MAT activity (MAT I/III deficiency) contain mutations in their *MAT1A* gene encoding the major hepatic forms, MAT I and III. MAT I/III deficiency is characterized by isolated persistent hypermethioninemia and in some cases, unusual breath odor. Several hypermethioninemic individuals have developed abnormal neurological problems including brain demyelination. We demonstrated that the brain demyelination patients are homozygous for *MAT1A* mutations that generates a truncated MATα1 subunit which is enzymatically inactive. We further showed that isolated persistent hypermethioninemia is a marker of MAT I/III deficiency, and that complete lack of MAT I/III activity can lead to neurological abnormalities.

Others:	Chi-Jiunn Pan	Chemist	HDB, NICHD
	Brian C. Mansfield, Ph.D.	Adjunct Professor	
	Baochuan Lin	Adjunct Student	HDB, NICHD

Project Description

Objectives:

The objectives of this project are to understand the molecular genetics of glycogen storage disease type 1 (GSD-1) and methionine adenosyltransferase (MAT) I/III deficiency, and the physiological functions of pregnancy-specific glycoproteins (PSGs).

GSD-1 is caused by a deficiency in glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis. GSD-1 is phenotypically divergent and has been divided into four subgroups, which correspond to defects in the G6Pase catalytic unit (1a), the putative G6P translocase (1b), the putative phosphate/pyrophosphate translocase (1c), and the putative glucose translocase (1d). This laboratory has pioneered the molecular genetic studies of GSD-1a and established the molecular basis of this disorder. Recently, we have generated *G6Pase*-deficient mice and shown that the knockout mice manifest essentially the same pathophysiology as human GSD-1a, indicating that the G6Pase$^{-/-}$ mouse is a valid animal model for the study of GSD-1a. However, we detected no mutations in the *G6Pase* gene of GSD-1b and 1c patients, suggesting that these patients may be defective in the putative translocases. Current areas of GSD research include: (1) to pursue the *G6Pase*-deficient mouse model to increase our understanding of the biology and pathophysiology of this disorder, to delineate the mechanisms of G6Pase catalysis, and to develop future therapeutic approaches for GSD-1a; (2) to clone the G6P translocase hypothesized to be deficient in GSD-1b patients; (3) to delineate the structure-function relationship of G6Pase catalysis; (4) to characterize the molecular mechanisms underlying tissue-specific and hormone-dependent expression of the *G6Pase* gene. The goals of our study are to understand the pathogenesis of GSD-1, to develop novel therapeutic approaches for this disorder, to delineate the mechanism(s) of G6Pase catalysis, and to characterize the gene deficient in GSD-1b.

Hepatic MAT I/III encoded by the *MAT1A* gene is a key enzyme in transmethylation, transsulfuration and the biosynthesis of polyamines. MAT I/III deficiency is characterized by isolated persistent hypermethioninemia and, in some cases, unusual breath odor or brain demyelination. This laboratory has pioneered the molecular genetic studies of MAT I/III deficiency and established the molecular basis of this genetic disorder. We further demonstrate that isolated persistent hypermethioninemia is a marker of MAT I/III deficiency, and that complete lack of MAT I/III activity can lead to neurological abnormalities. Current areas of MAT research include: (1) to identify mutations in the *MAT1A* gene of individuals with isolated persistent hypermethioninemia and to correlate the specific defects with serum methionine levels and neurological manifestations; (2) to delineate the structure-function relationship of MAT catalysis. The goals of our study are to understand the pathogenesis of MAT I/III deficiency and to accurately and non-invasively diagnose hypermethioninemic individuals suspected of having abnormalities at the *MAT1A* locus to assess if therapy aimed at the prevention of neurological manifestations is warranted..

PSGs form a family of closely related proteins which are essential for pregnancy. Despite being present at high levels in the maternal blood, the roles of PSGs are unknown. The goals of this project is to identify and characterize the biological roles of the PSG and explore their clinical use, during pregnancy and for contraception.

Methods Employed:

The principal methods are: (1) all current methods in nucleic acid research, including mRNA and DNA isolation, cDNA library construction, Northern and Southern blot analyses, S1-mapping, ribonuclease protection analysis, polymerase chain reaction, *in situ* hybridization, nuclear run-off assays, CAT assays, gel retardation assays, footprinting assays, site-directed mutagenesis, DNA cloning, SSCP analysis, and DNA sequencing; (2) all current methods in tissue culture research; (3) protein isolation techniques, including gel filtration ion-exchange chromatography, and affinity chromatography; (4) enzyme assays, both spectrophotometric and radiometric methods; (5) radioimmunoassays; (6) establishment of differentiated mammalian cell lines by viral transformation; (7) immunoprecipitation, gel electrophoresis, and fluorography; (8) expression of cloned genes in the baculovirus system; and (9) gene targeting and the generation of knock-out mouse strains; (9) positional cloning.

Major Finding:

A. Glycogen Storage Disease Type 1

GSD-1 is caused by a deficiency of glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis catalyzing the terminal step in gluconeogenesis and glycogenolysis. Diagnosis of GSD-1a currently is established by demonstrating the lack of G6Pase activity in the patient's biopsied liver specimen. Recent cloning of the *G6Pase* gene and identification of mutations within the gene that cause GSD-1a allow for the development of a DNA-based diagnostic method. Using single strand conformation polymorphism analysis (SSCP) and DNA sequencing, we characterized the *G6Pase* gene of 70 unrelated patients with enzymatically confirmed diagnosis of GSD-1a and detected mutations in all except 17 alleles (88%). Sixteen mutations were uncovered which were shown to abolish or greatly reduce G6Pase activity and are therefore responsible for the GSD-1a disorder. R83C and Q347X are the most prevalent mutations found in Caucasians, 130X and R83C in Hispanics, and R83H in Chinese. The Q347X mutation has thus far been identified only in Caucasian patients, and the 130X mutation only in Hispanic patients. Our results demonstrate that the DNA-based analysis can accurately, rapidly, and non-invasively detect the majority of mutations in GSD-1a. This DNA-based diagnosis now permits prenatal diagnosis among at-risk patients and serves as a data-base in screening and counseling patients clinically suspected of having this disease.

At present, treatments of GSD-1a focus solely on controlling symptomatic hypoglycemia. Patients who follow careful metabolic control continue to have blood lactate, cholesterol, and triglyceride values above the normal range and kidney function eventually becomes compromised. It is not clear whether long-term complications including gout, hepatic adenomas, and osteoporosis will eventually develop in the patients despite good dietary intervention. To understand the pathogenesis of this disorder, to delineate the mechanisms of G6Pase catalysis, and to develop future therapeutic approaches, we constructed *G6Pase*-deficient mouse strains by gene targeting. We have shown that the G6Pase$^{-/-}$ mice manifest essentially the same pathophysiology as human GSD-1a patients, including hypoglycemia, growth retardation, hepatomegaly, kidney enlargement, hyperlipidemia, and hyperuricemia. Human GSD-1a patients who do not receive dietary treatment die early, usually in the teens, of liver and/or kidney complications. Histopathological examinations of the G6Pase$^{-/-}$ animals revealed marked glycogen storage in the liver and kidney, and fat deposition in hepatocytes. The G6Pase$^{-/-}$ mice survived an average of 4 to 5 weeks and necropsy analysis revealed evidence of severe liver and renal lesions. The extensive similarities between homozygous mutant animals and human GSD-1a patients indicate that the G6Pase$^{-/-}$ mouse is a valid animal model for the study of GSD-1a.

This well defined mouse model of GSD-1a provides an opportunity to examine the two theories proposed for G6Pase catalysis. The multi-functional conformation-substrate-transport model is supported by observations that G6P transport and hydrolysis appear synchronous. The multi-component translocase-catalytic unit model, however, is supported by observations that liver microsomes in GSD-1b and 1c patients have G6Pase activity but no substrate or product transport activity. To assess these two models, we examined microsomal G6P transport and hydrolysis in liver and kidney microsomes from G6Pase$^{+/+}$ and G6Pase$^{-/-}$ mice. We show that the knockout of the *G6Pase* gene destroys both G6Pase activity and G6P transport, providing direct evidence of the tight coupling between G6P transport and G6Pase activity. By examining G6Pase in the liver and kidney, the primary gluconeogenic tissues, we demonstrate that G6P transport and hydrolysis are performed by separate proteins. We therefore propose a modified translocase-catalytic unit model, in which G6Pase catalysis consists of two tightly coupled reactions, G6P transport and G6P hydrolysis. Inactivation of transport prevents substrate access to the hydrolytic center while inactivation of hydrolysis abolishes transport by uncoupling an essential link.

G6Pase is primarily expressed in the liver and kidney and liver play a major role in glucose homeostasis. In order to gain insight into the molecular mechanisms regulating liver-specific expression of the human *G6Pase* gene, we characterized *G6Pase* promoter activity in hepatic and nonhepatic cells. Transient expression assays demonstrate that the *G6Pase* promoter is active in hepatoma cells but not in choriocarcinoma or 3T3 cells. We also show that nucleotides -234 to +3 DNA region upstream of the transcription start site of the *G6Pase* gene direct optimal *G6Pase* expression in hepatocytes. Sequence analysis indicates the presence of multiple transcription factor binding sites within this region, including motifs for liver-enriched factors such as hepatocyte nuclear factor (HNF) 1 (-226/-212), HNF3 (-180/-174, -139/-133, -91/-85, and -81/-75), HNF4 (-240/-229 and -231/-220), and C/EBP (-130/-125). Deletion analysis revealed the presence of three activation elements at nucleotides -234/-212, -181/-125, and -124/-71 of the *G6Pase* promoter. Gel mobility shift assays showed that HNF1, HNF3γ, and C/EBPβ bind to their respective cognate sites. Cotransfection studies demonstrated that HNF1α (not HNF1β), and HNF3γ transactivate *G6Pase* expression, while C/EBPβ plays little role in its expression. Our results indicate that liver-specific expression of the *G6Pase* gene is positively controlled by liver-enriched transcription factors, HNF1α and HNF3γ.

B. Methionine Adenosyltransferase I/III Deficiency

Methionine adenosyltransferase (MAT) is a key enzyme in transmethylation, transsulfuration and the biosynthesis of polyamines. Three forms of MAT (I, II, and III) have been identified in mammalian tissues. MAT I and III, which are encoded by the single copy *MAT1A* gene, represent tetramers and dimers formed from identical α1 subunits and are synthesized primarily in the liver. Genetic deficiency of MAT I/III causes isolated persistent hypermethioninemia and, in some cases, unusual breath odor or neural demyelination. However, the molecular mechanism(s) underlying this deficiency has not been clearly defined. To study the molecular genetics of this disorder, we characterized the human *MAT1A* gene and showed that it is a single copy gene consisting of 9 exons and 8 introns. Using SSCP and DNA sequence analyses, we characterized the *MAT1A* gene in eleven individuals with isolated persistent hypermethioninemia, including two with brain demyelination. Mutations that abolish or reduce MAT activity were detected in the *MAT1A* gene of all eleven individuals, thus establishing the molecular basis of MAT I/III deficiency. Both patients with demyelination are homozygous for mutations that alter the reading frame of the encoded protein such that the predicted MATα1 subunits are truncated and enzymatically inactive. The product of MAT, adenosylmethionine (AdoMet), is the major methyl donor for a large number of biologically important compounds including the two major myelin phospholipids, phosphatidylcholine and sphingomyelin. Both are synthesized primarily in the liver. Our findings demonstrate that isolated persistent hypermethioninemia is a marker of MAT I/III deficiency, and that complete lack of MAT I/III activity can lead to neurological abnormalities. Therefore, a DNA-based diagnosis should be performed for individuals with isolated hypermethioninemia to assess if therapy aimed at the prevention of neurological manifestations is warranted.

Analyses of the *MAT1A* mutations in these eleven MAT I/III deficient individuals and the available parents indicate that these mutations are inherited as autosomal recessive traits. However, dominant inheritance of hypermethioninemia, also hypothesized to be caused by MAT I/III deficiency, has been reported in two families. We therefore characterized the *MATA1* genes in all available members of the two families. The only mutation uncovered in the affected members of the two families is a G→A transition at nucleotide 791 in one *MATA1* allele that converts an Arg at position 264 to a His (R264H). Substitution of Arg-264 with His (R264H, the naturally occurring mutant), Leu (R264L), Asp (R264D), or Glu (R264E) greatly reduced MAT activity and severely impaired the ability of the MATα1 subunits to form homodimers essential for catalytic activity. On the other hand, when Lys was substituted for Arg-264 (R264K), the mutant α1 subunit was able to form dimers that retains high MAT activity, suggesting that residue 264 is involved in the formation of inter-subunit salt bridge. We further demonstrate that the R264H mutation exerts a dominant effect by forming enzymatically inactive R264/R264H MATα1 heterodimers.

C. Pregnancy-Specific Glycoproteins and Alkaline Phosphatase

PSGs form a large family of essential pregnancy proteins, but their biological function is unknown. We have investigated whether one function of the PSG is to interact with cells of the maternal immune system. Normal human peripheral blood mononuclear cells, activated with phorbol ester, are shown to bind to purified placental PSG. This binding activity can be mimicked using a chemically synthesized peptide ligand containing the Arg-Gly-Asp (RGD) motif present in the N-terminal domain of PSG11s. The PSG11s receptors are present on cells of the myeloid cell lineage but not of the T-cell or B-cell lineages. The binding is mediated in part by the RGD motif and can be competed against by appropriate RGD-containing, but not RAD-containing ligands. Ligand binding requiring functional receptors with different binding characteristics have been demonstrated. The THP-1 receptor is identified by chemical cross-linking as a protein of 46 kDa, and affinity chromatography demonstrated the presence of three proteins of 32, 16.8, and 15.9 kDa, suggesting the receptor has multiple subunits.

Human placentas express high levels of the placental alkaline phosphatase (*PLAP*) gene and low levels of a highly related gene, germ cell AP (*GCAP*). Malignant transformation of the placenta is accompanied by a reversal of this pattern of expression. Three Sp1-binding GC-rich DNA elements (sites I, II, and III) located within the first 156 base pairs upstream of the *GCAP* gene have been shown to direct optimal *GCAP* gene expression in choriocarcinoma cells. We now show that the first 100 base pairs upstream of the *GCAP* gene, which contains sites I and II, constitutes a minimal *GCAP* promoter. The simultaneous presence of both sites I and II is necessary for *GCAP* expression and its induction by sodium butyrate. The *PLAP* promoter directs only a very low level of gene expression in choriocarcinoma cells; the expression does not respond to butyrate. The -100/-1 DNA regions between the *GCAP* and *PLAP* promoters differ by only 8 base pairs. However, the GC-rich stretches in sites I and II of the *GCAP* promoter are disrupted in the corresponding *PLAP* promoter. This disruption blocks or markedly reduces the binding of choriocarcinoma nuclear factors to the *PLAP* promoter, leading to a reduction in expression and a loss of butyrate response. We further demonstrate that nucleotides -75 to -58 in both AP promoters, which bind a human Y-box binding protein, appear to downregulate *GCAP* expression.

Significance to Biomedical Research and the Program of the Institute

GSD-1 is caused by a deficiency in G6Pase, the key enzyme in glucose homeostasis. This metabolic disease typically manifests during the first year of life with severe hypoglycemia and hepatomegaly. Moreover, individuals with GSD-1 exhibit growth retardation, enlarged kidneys, lactic acidemia, hyperlipidemia, hyperuricemia, and in adults, a high incidence of renal failure and hepatic adenomas. Currently, four GSD-1 subgroups exist which correspond to defects in the G6Pase catalytic unit (1a), the G6P (1b), phosphate/pyrophosphate (1c), and glucose (1d) translocases. Characterization of the human *G6Pase* gene and mutations that cause GSD-1a have established the molecular basis of this genetic disorder. The generation of *G6Pase* knockout mouse which mimics the pathophysiology of human GSD-1a patients provides a valid animal model to study the pathogenesis of this disorder, to delineate the mechanisms of G6Pase catalysis, and to develop future therapeutic approaches. Characterization of the genes for the putative translocases and the mutations causing GSD-1b and 1c are essential for understanding the molecular basis of these disorders and the mechanism of G6Pase catalysis.

Newborns are routinely screened for high levels of serum methionine as an indicator of homocysteinemia deficient in cystathionine ß-synthase. High serum methionine levels are also found in patients with tyrosinemia type I and liver diseases. A subset of children with high serum methionine have been found to have isolated persistent hypermethioninemia (MAT I/III deficiency) without any other metabolic abnormalities. Additional individuals with biochemical abnormalities suggestive of MAT I/III deficiency have been identified and some patients developed abnormal neurological symptoms and brain demyelination. Characterization of the *MATA1* gene and mutations that cause MAT I/III deficiency are essential for understanding the etiology of this disorder. The demonstration that isolated persistent hypermethioninemia is a marker for MAT I/III deficiency and the development of a gene-based diagnosis of this disorder will allow the hypermethioninemic individuals to be positively diagnosed to assess if therapy aimed at the prevention of neurological manifestations is warranted.

National statistics reveal that over 50% of pregnancies end in miscarriages or abortion. Therefore, understanding the role of pregnancy-associated protein factors in implantation, maintenance of pregnancy, and fetal development is fundamental to women's health.

Proposed Course

1. Identification of mutations in the *G6Pase* gene of GSD-1a patients and study of the structural-functional relationship of G6Pase catalysis.
2. Pursuing *G6Pase*-deficient mice to delineate the pathogenesis of GSD-1a.
3. Development of gene therapy for GSD-1a.
4. Delineation of the mechanisms of G6Pase catalysis.
5. Cloning of the gene defective in GSD-1b patients and establishment of the molecular basis of the 1b disorder.
6. Establishment of an accurate, non-invasive, and gene-based prenatal diagnosis of GSD-1a and potential carriers.
7. Identification of mutations in the *MATA1* gene of patients with isolated persistent hypermethioninemia and study of the structural-functional relationship of MAT catalysis.
8. Delineation of the functional roles of PSG during pregnancy.

Publications:

Chamberlin ME, Ubagai T, Mudd SH, Wilson WG, Leonard JV, Chou JY. Demyelination of the brain is associated with methionine adenosyltransferase I/III deficiency, J Clin Invest, in press.

Chen H, Lin B, Chen C-L, Johnson PF, Chou JY. Role of the Transcription Factor C/EBPβ in

Expression of a Rat Pregnancy-specific Glycoprotein Gene, DNA Cell Biol 1995;14:681-688.

Chen H-D, Chen H, Chou, JY, Gillis KD, Chen C-L. Characterization of a prolactin-synthesizing rat pituitary cell line immortalized by the temperature-sensitive mutant SV40, Chin J Physiol 1995;4:201-209.

Earley K, Luo W, Qiu Y, Thompson NL, Chou J, Hixson DC, Lin S-H. Identification of a new isoform of cell-cell adhesion molecule 105 (C-CAM), C-CAM4: a secretory protein with only one Ig domain, Biochem J 1996;315:799-806.

Lei K-J, Chen Y-T, Chen H, Wong L-JC, Liu J-L, McConkie-Rosell A, Van Hove JLK, Ou HC-Y, Yeh NJ, Pan LY, Chou JY. Genetic basis of glycogen storage disease type 1a: prevalent mutations at the glucose-6-phosphatase locus, Am J Hum Gen 1995;57:766-771.

Lei K-J, Chen Y-T, Pan C-J, Ward JM, Mosinger B, Lee EJ, Westphal H, Chou JY. Glucose-6-phosphatase dependent substrate transport in the glycogen storage disease type 1a mouse, Nature Genetics 1996;13:203-209.

Matern D, Niederhoff H, Brandis M, Chou JY. Verification of diagnosis in a 17-year old boy with clinical glycogen storage disease type 1a by mutational screening, J Inherit Met Dis 1996;19:205-208.

Park C, Chamberlin ME, Pan C-J, Chou JY. Differential expression and butyrate response of human alkaline phosphatase genes are mediated by upstream DNA elements, Biochemistry, in press.

Rutherfurd KJ, Chou JY, Mansfield BC. A motif in PSG11s mediates binding to a receptor on the surface of the promonocyte cell line THP-1, Mol Endocrinol 1995;9:1297-1305.

Ubagai T, Lei K-J, Huang S, Mudd SH, Levy HL, Chou JY. Molecular mechanisms of an inborn error
of methionine pathway: methionine adenosyltransferase deficiency, J Clin Invest 1995;96:1943-1947.

Patent

Chou JY, Lei K-J, Shelly LL. Glucose-6-phosphatase: the gene and protein and related mutations. Allowed May, 1995.

LCE

NICHD Annual Report
October 1, 1995 to September 30, 1996

Laboratory of Comparative Ethology

Introduction

The Laboratory of Comparative Ethology (LCE) carries out a program of basic research investigating behavioral, cognitive, social-emotional, and physiological development in humans and in nonhuman primates. Both genetic and environmental influences and their multiple interactions are studied using a comparative approach in order to characterize the origins, ontogeny, and outcomes of various behavioral phenotypes. Results of experimental studies of biobehavioral development in nonhuman primates are compared with findings from long-term prospective investigations of human infants and their families, as well as with data obtained by neuroscience techniques. Longitudinal designs are employed in most major studies in order to address issues of developmental continuity vs. change, and in many of these investigations a variety of both behavioral and physiological measures reflecting multiple levels of analysis are collected concomitantly. A major emphasis is placed on characterizing and understanding normative patterns of development so that deviant patterns can be readily identified and their consequences evaluated with respect to established norms.

The LCE consists of four sections. The Comparative Behavioral Genetics Section, headed by Stephen Suomi, investigates biological and behavioral development in selected nonhuman primate species by focusing on interactions between genetic and environmental factors that affect the course of individual developmental trajectories throughout the lifespan. The Developmental Neuroethology Section, headed by John Newman, uses neuroscience techniques to study brain mechanisms involved in the production of various types of primate vocalizations, to examine subtle acoustical differences in these characteristic calls, and to investigate their function in several primate species, including humans. The Child and Family Research Section, headed by Marc Bornstein, examines cognitive, language, and social-emotional development in human infants and children, with special emphasis on studying the relationships among early attentional processes, social stimulation from and interactions with caregivers, and subsequent cognitive and social behavioral capabilities. Finally, the Section on Social and Emotional Development, headed by Michael Lamb, studies the effects of different types of caregiving practices on human infant and toddler social and emotional development and cognitive competence. Particular attention is given to longitudinal approaches that transcend infancy, childhood, and adolesence and process-oriented studies of interactional styles, memories, and social perceptions.

Comparative Behavioral Genetics Section (CBGS)

Several studies carried out in the CBGS this past year examined the effects of specific early experiences on behavioral and physiological development in rhesus monkey infants and juveniles. Repeated sampling of saliva in nursery-reared infants from birth onward documented the emergence of the prototypical primate pattern of circadian rhythmicity in cortisol output by 3 weeks of age. However, by 6 weeks of age this initially robust circadian rhythm began to deteriorate in the nursery-reared rhesus infants. Moreover, by 2-3 months of age, their cortisol output was closely linked to specific routine activities within the nursery environment, including some that did not directly involve the infants themselves; such linkage had the effect of disrupting the normative circadian pattern of cortisol secretion. In succeeding months the adrenocortical response to these routine nursery activities became significantly blunted, and a more definitive circadian pattern of cortisol secretion re-emerged. Comparable developmental data regarding circadian rhythmicity and sensitivity of adrenocortical response to routine events have been collected from mother-reared infants (and from their mothers as well); those data are currently being analyzed.

A second set of studies compared and contrasted the behavioral and physiological consequences of two standardized nursery rearing procedures for rhesus monkey infants and juveniles: continuous access to like-reared agemates (peer-only rearing) vs. "only" 2 hours of access to peers 5 days per week, the rest of the time spent individually housed with inanimate surrogate "mothers" (surrogate-peer rearing), beginning at 30 days and continued through 6 months of age. Somewhat surprisingly, surrogate-peer reared monkeys exhibited significantly higher levels of locomotion and social play, and lower levels of infantile social clinging and self-directed behavior, than their peer-only reared counterparts; in this respect, surrogate-peer reared monkeys more closely resembled mother-reared infants growing up in naturalistic settings than did peer-only reared subjects. Moreover, surrogate-peer reared monkeys exhibited less extreme adrenocortical responses to standardized environmental challenges at 3, 4, and 5 months of age than peer-only reared subjects (and, indeed, even lower than mother-reared monkeys of comparable ages). While these findings, suggesting that "less is better" from the standpoint of exposure to peers, might seem counterintuitive, they are clearly consistent with attachment theory principles regarding separate, often incompatible, socialization roles for maternal-like and peer relationships.

A third study investigating the effects of differential early rearing experiences involved foster-rearing rhesus monkey neonates selectively bred for extreme (high vs. low) patterns of central monoamine metabolism (as assessed by CSF concentrations of 5-HIAA) with foster mothers who differed systematically in their relative sociality and impulsiveness, respectively. Given previous findings linking low CSF 5-HIAA levels with asocial, impulsive, and often destructively aggressive behavior in juvenile, adolescent, and adult monkeys, this cross-fostering design will enable substantial disentanglement of heritable and experiential influences; more importantly, it will facilitate identification and characterization of interactions among these various influences, which are typically dynamic and complex. Data collection for this prospective longitudinal cross-fostering study is currently underway, encompassing a variety of behavioral and physiological measures sampled repeatedly from the infants, their foster mothers, and their biological mothers.

Two other studies investigated relationships between individual differences in 5-HIAA levels and other specific aspects of rhesus monkey life. In the first, a significant negative relationship was found between CSF 5-HIAA concentrations and both overall activity levels and sleep latencies in rhesus monkey families living in indoor-outdoor pens. A second study documented seasonal variability in 5-HIAA levels in free-ranging juvenile, adolescent, and adult rhesus monkey males. CSF 5-HIAA concentrations were highest during autumn months, rising significantly from summer levels. For these wild-living rhesus monkeys, the autumn months encompass the annual breeding season, and perhaps not surprisingly, these seasonal changes in 5-HIAA levels were most pronounced in the adult males and least pronounced in the juveniles.

Analysis of behavioral and physiological data from a long-term prospective longitudinal study of individual differences in developmental trajectories within a cohort of rhesus monkey infants growing up in wild troops on Cayo Santiago island (PR) focused this past year on reactions to brief maternal separations associated with mating activities and to the birth of younger siblings. In general, the reactions to brief maternal separation were considerably milder during the second year of life than during the first, although individual differences in the relative severity of behavioral disruption were relatively stable across years. The birth of a sibling was associated with sharp decreases in time spent in contact with or near the mother and concomitant increases in passivity, but not in active distress behavior; these changes tended to be greater in male than in female juveniles. Analysis of morphometric and psychophysiological data collected from these juveniles during their annual capture for veterinary examination revealed stable gender and individual differences in weight, skinfold, heartrate patterns, and vocalizations during heartrate recording periods. These data represent the first demonstration of significant developmental continuities of individual differences in behavior and physiology during the first two years of life in rhesus monkeys growing up in the wild.

A second study involving the wild rhesus monkeys living on Cayo Santiago island focused on the relationship between individual differences in behavioral and physiological response to brief capture (for veterinary examination) and patterns of maternal behavior exhibited by multiparous adult females. Females exhibiting high and stable heartrates also tended to have relatively high plasma cortisol levels following capture, replicating previous field data collected from infants and juveniles, as well as adolescent males. These "high-reactive" females exhibited lower amounts of ventral-ventral and dorsal-ventral contact, and higher rates of initiated contact breaks with their infants than did "low-reactive" females; they also tended to groom other adult females more, and had their infants contact their nipples less often and take greater responsibility for maintaining proximity, than their low-reactive counterparts. These data provide the first empirical evidence that maternal behavior patterns are influenced by maternal temperament in wild-living groups of rhesus monkeys.

Another long-term study in which data collection was completed this past year examined neurochemical and psychophysiological concomitants of reconciliation behavior following aggressive exchanges among group-living rhesus monkey females. Whereas aggressive behavior was characterized by sharp increases in heartrate, reconciliation (i.e., affiliation with former opponents immediately after aggressive episodes) was associated with significant decreases in heartrate, relative to preconflict, conflict, and nonconflict matched control periods. Behavioral and neurochemical data from this study are still being analyzed.

Longitudinal study of capuchin monkey biobehavior development yielded several interesting findings regarding both species normative patterns and individual differences in response to environmental challenge. First, short-term (2 hr.) separations from familiar conspecifics produced adrenocortical responses in 6-month and 1-year-old capuchin monkeys that were basically intermediate in magnitude between species-normative separation-induced cortisol levels for rhesus monkeys and other Old World monkey species, on the one hand, and the extreme levels characteristic of squirrel monkeys, a New World monkey species like capuchins, on the other. However, baseline cortisol levels of these capuchin subjects more closely resembled Old World monkey norms, both of which were significantly lower than squirrel monkey baseline cortisol levels. Second, individual differences in adrenocortical response to separation were highly stable from 6 to 12 months of age, paralleling a wealth of data from rhesus monkeys and other macaque species. Third, these differences in adrenocortical response were associated with differences in activity state patterns recorded in the first 3 months of life. Finally, individual differences in cortisol levels during separation were themselves predictive of subsequent object manipulation and proto-tool using behavior during the second year of life, which, in turn, appear to be predictive of marked individual differences in complex tool-using propensities later in life. These data represent the first concrete identification of any factor that is predictive of the dramatic individual differences in tool-using abilities that characterize this unusual primate species.

Extensive research investigating the tool-using capabilities of capuchin monkeys was continued in FY96, expanding considerably the known range and complexity of tool-using tasks the species is able to master under captive living conditions, including the use of tools to facilitate capture of live prey (ants). In addition, a paradigm was developed that enabled groups of capuchins housed in adjacent pens to obtain and share highly desirable food items only if tools to obtain the food were also shared. Several monkeys in both groups mastered this complex tool-and-food-sharing task, providing the first evidence of tool-sharing in any other primate species besides humans and chimpanzees. The degree to which laterality (handedness) was involved in the various tool-using tasks was also investigated in several studies. In general, a greater right-handed bias was found in adult subjects than in juveniles, and this right-handed bias was significantly stronger for tasks requiring a bipedal posture than those that could be solved while sitting or maintaining a quadrapedal posture.

Finally, data from several field studies investigating the relationship between social structure, environmental context, and specific vocal patterns in several New World primate species were published in FY96, as were several theoretical and review chapters that examined parallels in biobehavioral development between human and nonhuman primates.

Developmental Neuroethology Section (DNS)

Research carried out in the DNS this past year continued its focus on studies of vocalizations and other social behaviors and their associated brain processes in several primate species. Previous work in this and other laboratories has suggested major roles for the neuropeptides oxytocin and vasopressin in mediating a variety of contact-oriented social behaviors in marmosets. An ongoing series of autoradiographic studies has involved the mapping of oxytocin and vasopressin receptor sites in the brains of male and female marmosets of differing developmental status. Research completed in FY96 used ligands for central oxytocin and vasopressin receptors to compete with a125I-labeled ligand selective for oxytocin receptors to determine the ligand specificity of the binding sites revealed by autoradiography. Several binding sites for the oxytocin ligand 125I OTA were found in both male and female juvenile and adult brains. The largest site is in and around the nucleus accumbens and olfactory tuberculum, appearing rostrally as a single field but separating into two fields more caudally. A second smaller and much more localized site appears near the nucleus basalis and lateral amygdala.

A second set of studies focused on oxytocin and vasopressin immunoreactive neurons in the forebrains of male and female marmosets. Brain sections were processed for vasopressin immunoreactivity (AVP-ir). A dense cluster of AVP-ir cells was found in the paraventricular nucleus of the hypothalamus and caudally in the periventricular nucleus. Another cluster of AVP-ir cells was found in the supraoptic nucleus. Scattered AVP-ir cells were found in the lateral hypothalamus, suprachiasmatic nucleus, and stria terminalis. This last structure was the only brain area to exhibit sexual dimorphism in number of AVP-ir cells, with males having more cells than females. AVP-ir fibers were found in the hypothalamus, as well as the nucleus accumbens, septum, diagonal band of Broca, lateral preoptic area, and stria terminalis. Another set of brain sections from the same marmosets was processed for oxytocin immunoreactivity (OT-ir). Scattered OT-ir neurons were found in the same 3 hypothalamic nuclei containing AVP-ir cells, as well as the stria terminalis, medial amygdala, and lateral hypothalamus. No OT-ir cells were found in the suprachiasmatic nucleus. Male and female brains did not differ in the number of OT-ir neurons in any area examined. Finally, brain sections from a subset of the subjects were processed for androgen receptor immunoreactivity (AR-ir). Cells that stained for AR-ir were found mostly in the cerebral cortex, preoptic area, hypothalamus, lateral septum, amygdala, and bed nucleus of the stria terminalis. Male brain sections had more AR-ir cells in the lateral septum, stria terminalis, and medial amygdala than female sections. An alternate set of brain sections from the same marmosets were processed for both AR-ir and AVP-ir or AR-ir and OT-ir, to determine the presence of co-localization of AR-ir with AVP-ir or OT-ir. There was a lack of AR-ir colocalization with AVP-ir cells in virtually all areas examined. Some AR-ir colocalization with OT-ir was found in the paraventricular nucleus of the hypothalamus. This is the first study of AVP and OT immunoreactivity in a New World primate, and the results show that the pattern of AVP-ir distribution in marmosets is different from that reported in macaques as well as in rats and may represent a transitional AVP cell distribution between rodents and Old World primates.

A third set of studies investigated the effect of social context on phee call production in common marmosets who differed in age and reproductive status. Previous work in the DNS has shown that the common marmoset phee call exhibits context- and gender-specific differences in acoustic structure. As one of the contexts in which phee calls are produced is related to group spacing ('territoriality'), it was of interest to determine the extent to which nonreproductive family members participate in this calling behavior while in their natal group, and whether their vocal behavior changes after removal from the natal group and pairing with a mate. The phee calls of pubescent marmosets were recorded and analyzed under these 2 social conditions, as well as those produced during brief social separations. Subjects were then removed from their natal groups and rehoused as nonfamilial heterosexual pairs in new cages. Within the natal group, phee calling by these subjects was sporadic at best. In contrast, all subjects made home-cage phee calls after pairing, and calling rates averaged 25 times higher than in the natal groups. Calling rates of phees made during brief periods of social separation from both the natal and newly paired housing conditions did not differ, suggesting that the change in vocal behavior from the home cage context was not merely the result of being older, nor were any sex differences in vocal behavior observed. These results suggest that the natal family environment has an inhibiting effect on the home-cage phee calling by nonbreeding family members. The rapidity with which newly paired animals began calling suggests that physiological and neuroanatomical substrates necessary for home-cage calling may be in place but remain suppressed until released by the appropriate social context.

A follow-up study of postpubescent marmosets examined the relationships between the social environment, endogenous hormone levels, and vocal behavior. Some male and female subjects were separated from family groups and rehoused with unfamiliar, opposite-sex conspecific in a neutral arena for 24 hours, while others were briefly separated from their family group then returned to their families. A third group of adolescents were each separated from families for 24 hours, while a fourth group of singly-housed adolescent males and females were briefly separated from and then returned to their respective home cages. Femoral blood samples were collected on the day prior to testing and at various times during and after separations. The results indicate that both males and females separated for 24 hours exhibited steadily increasing levels of serum cortisol, with no apparent sex differences. In contrast, no significant changes in cortisol levels were found in those subjects experiencing only brief separation. The vocal behavior of these groups contrasted rather strikingly with their cortisol responses. Subjects from all groups exhibited high levels of calling during the brief separations. Following 24 hours of separation, however, calling rates fell back to baseline levels, during the very time when cortisol values were highest. Vocalizations of animals paired with a new mate remained higher one day after pairing than prior to pairing, although cortisol levels in these paired animals did not change significantly.

Two additional studies completed this past year utilized a computer-based acoustical analysis system (LMA) developed by DNS personnel. In the first study, vocalizations were recorded from rhesus monkey infants at 1, 2, 3, and 4 weeks of age during routine Brazelton assessments; additional recordings were made during brief separations at 2, 3, 4, and 5 months in a second set of rhesus monkey infants. Each vocalization of suitable acoustic quality was digitized, then subjected to Fourier analysis using the SIGNAL program. The FFT files were then used to calculate a large number of acoustic parameters, using LMA. Based on these calculations, a cluster analysis demonstrated that the vocal subtype typically associated with social separation in the rhesus macaque, the “coo,” could be reliably distinguished from other vocal subtypes produced in the separation context. Subsequent analyses identified specific vocal characteristics associated with individual differences and the age of the vocalizer. In general, coos had a flatter slope, less modulation, and a slightly lower pitch with increasing age. These analyses are important, because they provide the basis for future examination of biologically relevant variables, such as type of rearing, individual experience, and measures of temperament or emotionality, in the vocal patterning of rhesus monkey infants.

A second study utilizing the LMA acoustical analytical system involved a sample of human nursing home residents at the Hebrew Home of Greater Washington, in Rockville, MD. The sample included 25 residents (20 female, 5 male) with a mean age of 86.1 years, from whom repetitive vocalizations were recorded either alone or in a large room with other residents present. All 25 residents had been diagnosed as having dementia, including some with Alzheimer’s disease, vascular dementia, or dementia of unknown origin. The vocalizations were subjected to Fourier analysis, and 85 acoustic parameters were calculated using LMA. A discriminant function analysis correctly classified over 90% of the cases into the correct dementia categories on the basis of acoustic differences. This finding is remarkable, given the high degree of individuality in the vocal utterances. The potential clinical value of the findings from this study is in the prospect of using the acoustic character of the vocalizations from aged individuals to make a preliminary diagnosis as to the presence of dementia and its possible etiology. It also represents an example of the usefulness of an analytical approach developed for nonhuman primate vocal behavior in assessing clinically relevant aspects of human vocal behavior.

Child and Family Research Section (CFRS)

Research carried out in the CFRS this past year continued a series of longitudinal investigations of the development of attentional, cognitive, and personality characteristics, the influence of various aspects of maternal care, and the emergence of play and language and their relationship to representational capabilities in human infants and children over the first 4 years of life. These investigations utilized both normative and clinical samples of children and their mothers from a variety of sites both within the U.S. and abroad. Standardized assessments of child capabilities and maternal characteristics at common age points (e.g., 5, 20, and 48 months) in most of the samples have permitted direct comparisons of various aspects of child development both within and between a variety of distinctive cultures.

Several sets of analyses completed in FY96 focused on normative aspects of attentional, language, play, and representational development. These analyses (a) described age-related changes in each of these domains; (b) characterized the nature and extent of individual and gender-related differences at each age tested; (c) identified correlates of those individual and gender-related differences in other infant domains, as well as with various characteristics, beliefs, and behaviors of the mothers; and (d) examined the extent to which measures within or correlates of each domain at early ages were predictive of specific capabilities later in childhood. For example, multiple measures of overall language competence (e.g., language comprehension, word production, and language expression) were highly intercorrelated at 20 months of age, tended to be higher in girls than in boys, and showed considerable variation within both genders at this age. Language competence at 20 months was directly and positively related to the child's overall social competence, as well as with the mother's language competence and knowledge of and attitude toward parenting; child language competence at 20 months was also indirectly influenced by both sociodemographic and personological characteristics of the mother. Other analyses explored the relationship between child language competence at 20 months and various types of play behavior, both alone and with the mother. Structural equation modeling revealed that child language competence, along with the amount of symbolic play by the mother, were predictive of the amount and quality of collaborative play exhibited by the child at 20 months, whereas solitary play by the child was predicted by child gender and mother's verbal intelligence and physical affection, which in turn, influenced mother's play and so influenced child collaborative play indirectly.

The predictive power of measures of child language competence at 20 months was demonstrated in follow-up analyses of the children's representations of affectively-neutral and affectively-laden events at 48 months of age. This relationship was of interest for several reasons: First, affect has been found to play a role in the acquisition of language and of other aspects of language-based representational competence. Second, children begin to use their experiences of routine events to organize their real-world knowledge into event representations by around 2 years of age, concurrent with toddler language development. Third, interpersonal affect and emotion language are involved in the co-construction of an event and therefore the event's representation. Preschoolers' reports of an affectively-laden event (a visit to the doctor) were more likely to contain affective content than their reports of an affectively-neutral event (description of lunch). A model of sociodemographic variables and toddlers' language capabilities as predictors of prescshoolers' verbal reports of affectively-neutral and affectively-laden routine events was tested using two hierarchical multiple regression analyses, each controlling for sociodemographic variables known to influence language development (SES and maternal IQ). These analyses indicated that SES was the sole predictor of the number of acts in children's reports of the affectively-neutral lunch event. However, mothers' reports of their children's language production at 20 months significantly predicted the number of acts reported in the affectively-laden doctor event. Together with analyses indicating that children are significantly more likely to include affective content in their verbal reports of going to the doctor, these results suggest that child affect and language may play important roles in the acquisition, organization, and narration of specific routine events.

The availability of an extensive longitudinal data base documenting normative aspects of attentional, cognitive, language, and affective development has also facilitated investigations involving specific clinical populations of infants and children. Most studies of mothers who have children with disabilities tend to view them as a homogeneous group with a distinct interaction style, rather than individuals whose styles vary from maternal behaviors of mothers of normally developing children depending on many circumstances. In contrast, a study completed by CFRS staff and collaborators this past year was organized to document the influences of the loss of a major communication channel (deafness) on mother-child interaction and on children's developing language and play performance during the second year of life in order to explore the role of children's early language learning environments on the development of symbolic activity. Three independent measures of child language competency were obtained, and all three yielded similar results: The two groups of hearing children (with hearing mothers and with deaf mothers) had the highest means and were nearly indistinguishable, the deaf children of hearing mothers had the lowest mean for each language variable, and the deaf children of deaf mothers fell between these extremes. Deaf children's language delays, unlike those of most other groups of children with disabilities, usually occur because of lack of access to a language model and are not necessarily associated with neurological or cognitive developmental difficulties. Not surprisingly, deaf children with hearing parents had high rates of language delays and deficiencies. By contrast, no overall group differences emerged for frequency of symbolic play in either the solitary or collaborative play conditions.

A second set of investigations involving a clinical population focused on children of cocaine-abusing mothers. Previous work by the CFRS, in collaboration with the Yale Child Study Center, has demonstrated that infants exposed to cocaine in utero exhibit some deficits in arousal regulation and motor development, but relatively normal rates of habituation and recovery of attention to novel stimuli by 3 months of age, suggesting that the effects of prenatal cocaine exposure are largely centered on arousal and attention regulation rather than on more general cognitive processes in early infancy. Research this past year focused on how cocaine use affects mothers' attentiveness and responsiveness to their infant in face-to-face interactions. At 3 and 6 months, cocaine-using mothers were less attentive to interactions, and cocaine-using mothers and their infants engaged in fewer dyadic interactions, than either non-cocaine or non-drug-using mothers. Compared to 3 months, cocaine-using mothers at 6 months were less attentive to interactions and more frequently interrupted interactions by looking away, redirecting the infant, or withdrawing, whereas non-cocaine-using and non-drug-using mothers showed no change or an improvement in attentiveness to interactions and a decrease in interruptions. No differences emerged in the interactive behaviors of the infants of cocaine-using, non-cocaine-using, or non-drug-using mothers, but by 6 months, the infants of cocaine-using mothers were less interactive than either the infants of non-cocaine-using or non-drug-using mothers. Cocaine use thus appears to represent a significant risk for diminished parental attentiveness and responsiveness to infants. Current work is focused on examining the potential longer-term effects of prenatal cocaine exposure and continued cocaine used by mothers on developmentally salient tasks and behaviors in the toddler and preschool periods. Preliminary analyses of 18-month-old children's play activities with their mothers indicated no significant differences in the frequency or duration of symbolic play by mothers or children among groups of cocaine-exposed, other-drug exposed, and non-drug-exposed children. Analyses are proceeding to determine whether differences exist between these groups in patterns of mother-child dyadic interaction during play, as reflected in the emotional availability of mothers and children, or in children's language acquisition and use.

Analyses of data collected from the CFRS's numerous cross-cultural studies of development this past year centered largely on comparisons of parental beliefs, expectations, and practices. In one study, ideas that Argentine, French, and U.S. mothers hold about their own and their husbands' actual childrearing, as well as what mothers consider to be ideal childrearing, were investigated in three domains: social, didactic, and limit-setting. For each domain, the analyses compared mothers' reports of their actual behaviors and of their husbands' actual behaviors; mothers' ideal expectations of their own and their husbands' behaviors; and mothers' dissonance with respect to parenting, i.e., the extent to which mothers' actual and their husbands' actual behaviors were consistent with mothers' ideal expectations. The results showed consistent parent, country, as well as parent-by-country effects interpretable in terms of overarching cultural beliefs. A second study compared beliefs about parenting in Argentine, Belgian, French, Israeli, Italian, Japanese, and U.S. American mothers of 2-year-olds. Mothers evaluated their competence, satisfaction, investment, and role balance in parenting, and attributed successes and failures in parenting to ability, task difficulty, child behaviors, effort, and mood. Systematic country effects for both self-perceptions and attributions emerged; these results were interpreted in terms of specific cultural proclivities and emphases.

In a third set of cross-cultural comparisons, psychosocial predictors of Argentine and U.S. primiparous mothers' decisions to work and amount of hours worked (in working mothers) were examined when their infants were 5 months old. Results revealed that years of marriage and months that mothers worked during pregnancy predicted whether or not mothers were employed after childbirth in both settings. By contrast, when the number of hours that mothers worked was analyzed, different results were found in the two countries: Better educated women with higher status occupations worked longer hours after childbirth in the United States, whereas better educated women with higher status occupations worked fewer hours in Argentina. Different cultural and economic conditions in the two countries appeared to mediate psychosocial determinants of women's decision to work.

Three additional analyses compared mother-infant interactions from two different cultural niches within Italy, one in an urban region in Northern Italy, the other a rural setting in Southern Italy. In the first analysis, maternal speech to 13-month-old infants was examined to verify how cultural variation influences the contents of that speech in terms of affect-salient and information-salient components. The results showed similarity in amounts of affect-salient contents of the speech in the two samples: Mothers seem to share feelings and to contribute to emotional exchange via their affect-salient speech to babies. However, differences in the two samples appeared in the contents of information-salient language. A second analysis examined how intranational cultural variation might influence the ways in which mothers use language pragmatically when addressing their 13-month-old children. It was hypothesized that mothers in the rural context would adopt speech acts with a control function more than mothers in the urban context, whereas urban mothers would use speech acts with a didactic function more than rural mothers. The results showed both intracultural similarities in some maternal communicative functions (Tutorial and Asynchronous) and significant intracultural differences in other communicative functions (Didactic and Control). These latter are presumably related to different life environments and childrearing practices, as reported by parents in a questionnaire. A third analysis compared mothers and their 20-month-old children from the same two sociocultural contexts with respect to the level of play shown in a naturalistic play session. The analyses revealed no differences between the two samples of children regarding the frequency and the duration of non-symbolic and symbolic play. Thus socio-cultural context seems not to influence non-symbolic or symbolic play in the South versus in the North of Italy, despite the regional differences in the aspects of maternal speech described above.

A final area of research carried out in the CFRS this past year examined the contributions of both child developmental and individual-difference factors and maternal perceptions on the utilization of health care systems. One set of analyses focused on 41 firstborn children for whom complete medical records were available for the full 12-month period immediately prior to a 4-year laboratory follow-up visit. Sociodemographic characteristics (including maternal age and level of education), maternal anxiety about child health, and past pediatric health care utilization (as documented in children's own medical records) were evaluated as predictors of mothers' general and specific ideas about their children's susceptibility and their own propensity to seek pediatric care. Results indicated that mothers simultaneously possess general and specific ideas about their children's susceptibility to illness and their own propensity to seek pediatric care, and that these general and specific ideas are differentially predicted by maternal anxiety and the number of recent pediatric visits made by children.

Finally, several major theoretical and review papers were published this past year, as well as a 5-volume handbook on parenting, an edited volume on child development and behavioral pediatrics, and a UNICEF technical report.

Section on Social and Emotional Development (SSED)

Research carried out in the SSED this past year centered on 4 major areas of developmental study. One long-term longitudinal project involved detailed descriptions of early parent-infant interactions in diverse ecological contexts. In this project mothers' diary-based descriptions of infant-related activities during the previous 24-hour period, long-term (e.g., 4-hr.) home observations of parenting activities, and laboratory-based assessments of infant capabilities and mother-infant interactions have been collected from a sample of upper-middle class Euro-Americans and lower-class Central American families living in the Washington, D.C. area, as well as from Costa Rica, Quebec, Germany, the Central African Republic, and Colombia. Analyses completed this past year focused on comparisons of mothers' reports of daily activities and their socialization goals between the two Washington, D.C. samples when the study infants were 8 and 12 months old. Results indicated that, in general, the infants' experiences and activities and the effects of the mothers', fathers', or others' presence on ongoing activities were all highly similar in both groups. The groups differed, however, with regard to the circadian distribution of activities, opportunities for interactions with various people, and the differences between weekdays and weekends. Overall, the social worlds of the Central American children were characterized by the simultaneous presence of several people and thus by multiple social partners, whereas the social worlds of the Euro-American children were characterized by more opportunities for dyadic interactions and by exposure to fewer partners.

A subsequent analysis investigated the relationship between mothers' long-term socialization goals and their evaluations of infants' behavior in a variety of everyday contexts, including feeding, sleeping, playing, and diaper changing. In terms of their long-term socialization goals, the Central American mothers stressed a desire for their children to learn proper demeanor and respect for authority, whereas the Euro-American mothers stressed the importance of fostering autonomy and independence. Examination of mothers' evaluation of infant behavior indicated that the Central American mothers placed greater weight on the appropriateness of the infants' behavior in each of these contexts, whereas the Euro-American mothers focused more on external factors such as the presence of others, location, and timing. These findings suggest that the extent to which infants are considered responsible partners in the smooth functioning of everyday activities varies culturally and relates coherently to culture-specific emphases on interdependence or independence. Comparable analyses of data collected from the Costa Rican and German sites are currently underway.

A second major area of study in the SSED continued long-term follow-up investigation of a Swedish sample of children who had been raised since 16 months of age in a center-based day-care setting, in a home-based day-care setting, or at home with one or both parents. Previous analyses of the longitudinal data collected on these children from toddlerhood through entry into the first grade consistently demonstrated that while the type of child care had little or no differential impact on any measures, the quality of home care and quality of alternative care had powerful effects on emerging social skills, personal maturity, and behavior in compliance-eliciting settings. During the past year the association between child-care arrangments and subsequent cognitive and personality development was examined. Parental ratings of field independence, ego resilience, and ego-control obtained using the California Q-set when the children averaged 28, 40, 80, and 101 months of age revealed virtually no differences between the developmental trajectories of the children in home care and in center-based daycare. By contrast, ego-undercontrol decreased less, while ego-resilience and field independence increased less, in children who attended family daycare than in the children in either center care or exclusive parental care. The quality of home and out-of-home care, as well as the socioeconomic status and family background, did not qualify these effects of the type of care experienced. Other analyses of the same data were designed to evaluate the Five Factor Model of personality. The factors identified by John et al. in a study of adolescents were barely reliable when the children averaged 29 months of age, but were sufficiently and increasingly reliable in the assessments which took place when the children averaged 40, 80, and 101 months of age. Results of a cross-validation analysis, employing data obtained when the children were in second grade, indicated that the factors were meaningfully related to measures of the children's cognitive abilities and school adjustment at these later ages.

The diverse developmental trajectories of the children in the home-care and the center-based daycare groups also differed with respect to the development of verbal abilities. When the children were eight years old and in the second grade of elementary school, tests and teachers' ratings of the children cognitive abilities were administered to the 123 children (65 girls) remaining in the study. Tested ability was related to the number of months children had spent in center-based day care before 3 ½ years of age. Structural and dynamic aspects of the child care quality also predicted cognitive abilities among those children who had spent at least 36 months of their preschool years in out-of-home care. Both measures of cognitive abilities were related significantly to preschool measures of verbal ability and to paternal involvement during the preschool years.

A third major program of research in the SSED concerned the emergence of gender differences in attitudes, expectations, and attributions, from the perspective of both children and adolescents and their parents. Analyses of data from two long-term projects were completed in FY96. The first project utilized interviews of three generations of mothers in Palestine to explore transgenerational changes in childrearing practices and beliefs in a culture exposed to enormous exogenous influence over the past half-century. These mothers, grandmothers, and great-grandmothers, all from the same Arab village, had surprisingly similar views of gender-differentiated practices and behavior, with mothers in all three cohorts maintaining traditional views and practices. As predicted, maternal ideas and socialization practices were found to be different for boys and girls on several variables: age of playmates, types of daily activities, location of daily activities, maternal satisfaction with children's caretaking, maternal dissatisfaction with children's lying and wandering, importance attached to marriage, ideal age of marriage, and ideal number of boys versus girls in the family. Contrary to prediction, however, differences were found on only a few variables among the ideas and socialization practices of mothers from the three generations: children's engagement in watching television, location of daily activities, maternal reactions to girls' caretaking of their siblings, and maternal perceptions of the ideal age for marriage. Thus, contrary to expectations, it did not appear that contact with the modern Israeli society has affected the basic values and beliefs of women in this one Arab village.

The second project assessed gender differences in the self-perceptions of a sample of 7th and 10th grade adolescents in the Washington, D.C. area. In the study, internally reliable multi-item measures of affiliation, assertion, and negative affiliation (traits that interfere with closeness) were constructed to assess whether self-ratings varied according to gender, age, and/or role. As predicted, girls reported being significantly more affiliative than did boys, whereas boys rated themselves higher on the negative affiliative scale. There was no evidence that gender differences increased from 7th to 10th grade. Contrary to prediction, there was little evidence of gender differences for assertion when combined role ratings were employed. However, the effect of gender was significant when assertion was analyzed according to role. Although there were no gender differences for 10 of 11 roles, boys' self-ratings on the assertive scale were higher than girls' for "myself as a boy/girl." Self-ratings varied significantly according to role on all 3 scales, and sex-role interactions were also significant. Boys' and girls' self-ratings tended to change in opposite directions when they pictured themselves in gendered roles: "Myself as a Boy" was the most assertive and the most negative affiliative role; in contrast, girls' ratings on these scales were higher for "Myself as I Usually Am."

The fourth program of research carried out in the SSED this past year explored the long-term effects of domestic vioence on children who were witnesses of spousal abuse and/or victims of physical abuse themselves, as well as continuing to develop and assess techniques for enhancing the informativeness of child witnesses and for evaluating the credibility of their accounts of abuse. Previous work in the SSED has demonstrated that the effects of domestic violence on child development vary in nature depending on the type of violence and the source of information about the child's adjustment. However, girls appear to have significantly more adverse effects overall from these experiences than do their male counterparts. A long-term follow-up study of the original child witnesses and victims completed this past year analyzed the children's account of conflicts with their parents in order to explore whether (a) greater reliance on over-general memory retrieval was related to depression, and (b) domestic violence affected the specificity of children's memory retrieval. As predicted, there was a positive correlation between children's 'generic-categoric' memory retrieval and their depression level. Children who scored higher on depression gave significantly higher proportion of 'generic-categoric' responses to questions concerning child-parent and inter-parental disagreements. However, there was no evidence that autobiographical memory was affected by domestic violence.

The other major focus of this research program reflected efforts to evaluate and improve upon the Criterion-Based Content Analysis (CBCA) system for eliciting and assessing statements by children alleged to have been victims or witnesses of sexual abuse. This past year transcripts of forensic interviews with alleged victims of child sexual abuse were scored for the presence or absence of the CBCA criteria believed to be more characteristic of accounts concerning experienced than nonexperienced events. As predicted, more of the CBCA criteria were present in accounts independently rated as Likely or Very Likely to have occurred than in accounts of events deemed Unlikely or Very Unlikely to have occurred. Several of the criteria were especially helpful in distinguishing between plausible and implausible accounts, but the group differences were not as dramatic as those reported in earlier studies. Other analyses confirmed and replicated the prediction that open-ended invitations yielded significantly longer and more detailed responses than directive, leading, or suggestive utterances regardless of age and culture (Israel, United States). Younger children provide briefer and less detailed responses, however. Detailed psycholinguistic analyses of interviews of 4- to 12-year-old children by police investigators in the United States revealed that the superiority of invitations was greater when the children reported experiencing three or more, rather than only one, incidents of abuse. Invitations were rarely used, however, and the investigators often failed to elicit more information from children who reported multiple incidents of abuse than from children who reported only one incident.

A subsequent experimental study evaluated the relative effectiveness of two techniques for motivating young witnesses, ranging in age from 4 to 12 years, to provide detailed accounts of alleged experiences of sexual abuse. In half of the interviews, investigators who were naive with respect to the experimental hypotheses used a script containing many open-ended utterances to establish rapport, whereas in the other interviews the same investigators used a script involving many direct questions. Both introductory scripts took about 7 minutes to complete and both included the identical open-ended statement to initiate the substantive phase of the interview. Children who had been trained in the open-ended condition provided 2 1/2 times as many details and words in response to the first substantive utterance as did children in the direct introduction condition. Children in the open-ended condition continued to respond more informatively to open-ended utterances in the later (unscripted) portion of the interview. Seventy-five percent of the children mentioned the core details of the incident in their responses to the first substantive utterance and a further 20% mentioned core details more vaguely. A final study completed this year compared investigative interviews in which anatomical dolls were employed for the purposes of demonstration with another in which the dolls were not used. Cases were matched with respect to other characteristics of the children and the alleged events. Children interviewed with dolls provided an equivalent number of details and spoke as many words in the substantive portions of the interview as did children interviewed without dolls, and interviewers in the two groups used similar probes to elicit information. The average responses by the children were significantly longer and more detailed when dolls were not used, however. Moreover, children gave longer and more detailed responses to open-ended invitations when dolls were not used.

Finally, several major theoretical and review papers were authored this past year by SSED staff. In addition two edited volumes on paternal beahvior and cross-cultural comparisons of childhood were published.

LABORATORY OF COMPARATIVE ETHOLOGY, NICHD

Chief, *Stephen J. Suomi, Ph.D.

Senior Staff
*Marc H. Bornstein, Ph.D.
*Michael E. Lamb, Ph.D.
*John D. Newman, Ph.D.

Staff Scientist
O. Maurice Haynes, Ph.D.
Kathleen T. Sternberg, Ph.D.

Fellows
Carol Andreassen, Ph.D.
Cecile Bassen, M.D.
Gayle D. Byrne, Ph.D.
Maribeth Champoux, Ph.D.
Janice Genevro, Ph.D.
Kathleen Moody, Ph.D.
Janet Norcross, Ph.D.
Samia Noursi, Ph.D.
Yael Orbach, Ph.D.
Kathlyn Rasmussen, Ph.D.
Gregory Westergaard, Ph.D.

Visiting Program
Kurt J. Hammerschmidt, Ph.D.
Birgit Leyendecker, Ph.D.
Angelika Timme, Ph.D.
Holger Wessels, Ph.D.
Kim Roberts, Ph.D.

Guest Researchers
Kathryn Bayne, Ph.D.
Olga V. Bazhenova, Ph.D.
Maxeen Biben, Ph.D.
Evan A. Byrne, Ph.D.
J. Dee Higley, Ph.D.
Ajit Maiti, M.D., Ph.D.
Melinda A. Novak, Ph.D.
Eric Phoebus, Ph.D.
Stephen Porges, Ph.D.
Axel Scholmerich, Ph.D.
Susan Theut, Ph.D.
Andra M. Rose, Ph.D.

LCE BIBLIOGRAPHY

October 1, 1995 to September 30, 1996

Altshuler JL, Genevro JL, Ruble DN, Bornstein MH. Children's knowledge and use of coping strategies during hospitalization for elective surgery. J Appl Develop Psych 1995;16:53-76.

Andreassen C, Bornstein MH. An invitation to children's storytelling. Review of Relating Events in Narrative: a Crosslinguistic Developmental Study by RA Berman, DI Slobin. Contemporary Psychology 1996;

Baumwell L, Tamis-LeMonda C S, Bornstein MH. Styles of maternal interaction and toddler language comprehension: The importance of maternal sensitivity. Inf Behav Develop 1996;

Berman CM, Rasmussen KLR, Suomi SJ. Group size, infant development, and social networkds: A natural experiment with free-ranging rhesus monkeys. Animal Behav, in press.

Boinski S. Stress responses in primates: Proximate mechanisms in the evolution of social organization. In: Foster SA, Endler JA eds. Geographic variation in behavior: An evolutionary perspective. Oxford: Oxford University Press, in press.

Boinski S, Campbell AF. Use of trill vocalizations to coordinate troop movement among white-faced capuchins: A second field test. Behaviour, in press.

Bornstein MH. Form and function: Implications for studies of culture and human development. Culture & Psychol 1995;1:123-37.

Bornstein MH. ed. Handbook of Parenting.
Volume 1: Children and Parenting.
Volume 2: Biology and Ecology of Parenting.
Volume 3: Status and Social Conditions of Parenting.
Volume 4: Applied and Practical Parenting.
Mahwah, NJ: Lawrence Erlbaum Associates, 1995.

Bornstein MH. Pomiędzy opiekunami a ich potomstwem: dwa rodzaje interakcji I ich konsekwencje dlarozwoju poznawczego. In: Brzezińskiej A, Lutomskiego G, Smykowskiego B, eds. Dziecko Wśród Rówieśników I Dorosłych [The Child in the World of People and Objects]. Poznań, Poland: Zysk i S-ka Wydawnictwo, 1995;39-63

Bornstein MH. Parenting infants. In: Bornstein MH, ed. Handbook of Parenting, vol 1. Mahwah, NJ: Lawrence Erlbaum Associates, 1995;3-39.

Bornstein MH. Origins of communication in infancy. In: Velichovsky BM, Rumbaugh DM, eds. Human by Nature: Origins and Destiny of Language. Mahwah, NJ: Lawrence Erlbaum Associates Publishers, 1996;139-72.

Bornstein MH. Parenting young children. In: Dalais C, Landers C eds. Child in Focus. New York: UNICEF, 1996.

Bornstein MH. La relazione madre bambino in una prospettiva cross-culturale. Somiglianze e specificità culturali in differenti contesti: Americhe, Giappone, Europa. [Mother-infant relationships in cross-cultural perspective: Similarities and specifics in different cultural settings in the Americas, Japan, and Europe]. In Venuti P, Giusti F, eds. Madre e padre: scienze dell'evoluzione, antropologia e psicologia delle funzioni parentali. Florence, Italy: Giunti Gruppo Editoriale, 1996,119-137.

Bornstein MH. Stability in mental development from early life: methods, measures, models, meanings, and myths. In: Butterworth GE, Simion F, eds. The development of sensory, motor & cognitive capacities in early infancy: from sensation to cognition. Hove: Erlbaum (UK) Taylor & Francis, 1996;

Bornstein MH. Parenting. In Friedman HS, ed. Encyclopedia of Mental Health. San Diego, CA: Academic Press, 1996;

Bornstein MH. Parenting young children. UNICEF Technical Report, 1996.

Bornstein MH, Bornstein HG. Infancy. In: Bornstein MH, Bornstein HG, Landers C, eds. Infancy and Early Child Development. Florence, Italy: UNICEF Innocenti Centre, 1996;

Bornstein MH., Bornstein HG. Caregivers' responsiveness and cognitive development in infants and toddlers: Theory and research. In: Mangione PL, ed. Infant/Toddler Caregiving: A Guide to Cognitive Development and Learning. Sacramento, CA: California Department of Education's Bureau of Publications, 1995;12-21.

Bornstein MH, Bornstein HG, Landers C. Infancy and Early Child Development. Florence, Italy: UNICEF Innocenti Centre, 1996.

Bornstein MH, Brown EM, Slater AM. Patterns of stability and continuity in attention across early infancy. J Reprod Inf Psychol 1996;14:195-206.

Bornstein, MH, Genevro JL. eds. Child Development and Behavioral Pediatrics.
Mahwah, NJ: Lawrence Erlbaum Associates, 1996.

Bornstein MH, Genevro JL. Child development and behavioral pediatrics: An introduction. In: Bornstein BH, Genevro JL, eds. Child Development and Behavioral Pediatrics. Mahwah, NJ: Lawrence Erlbaum Associates, 1996;1-9.

Bornstein MH, Haynes OM, O'Reilly AW, Painter K. Solitary and collaborative pretense play in early childhood: sources of individual variation in the development of representational competence. Child Develop 1996;

Bornstein MH, Maital S, Tal J, Baras R. Mother and infant activity and interaction in Israel and in the United States: a comparative study. Internat J Behav Develop 1995;18:63-82.

Bornstein MH, Mayes LC., Tamis-LeMonda CS. Habituation, information processing, mental development, and the threat of cocaine exposure in infancy. In: Hepper PG, Kendal-Reed M, eds. Perinatal Sensory Development: Psychology and Psychobiology. Cambridge, England: Cambridge University Press, 1996;xx-xx.

Bornstein MH, Slater A, Brown E, Roberts E, Barrett J. Stability of mental development from infancy to

later childhood: three "waves" of research. In: Bremner G, Slater A, Butterworth GE, eds. Infant Development: Recent Advances. Hove, England: Lawrence Erlbaum Associates, 1996;

Bornstein MH, Tamis-LeMonda CS. Parent-child symbolic play: Three theories in search of an effect. Develop Rev 1995; 15:382-400.

Bornstein MH, Tamis-LeMonda CS. Des vocalisations prélinguistiques à la communication linguistique: facteurs langagiers et non langagiers. [Language and nonlanguage factors in development of prelinguistic vocalization to linguistic communication.] In: Konopczynski G, Vinter S, eds. Le Développment Langagier: Une Prédiction Précoce Est-elle Possible? [Language Development: Is Early Prediction Posible?]. . Calais, France: Ortho-Edition, 1995;81-102.

Bornstein MH, Tamis-LeMonda CS, Pascual L, Haynes OM, Painter K, Galperín C, Pêcheux M-G. Ideas about parenting in Argentina, France, and the United States. International Journal of Behavioral Development 1996;

Boyce WT, Higley JD, Jenerin J, Champoux M, Suomi SJ. Tympanic temperature asymmetry and stress behavior in rhesus monkeys and children. Arch Ped Adol Med, in press.

Broberg AG, Wessels H, Lamb ME, & Hwang CP. The effects of day care on the development of cognitive abilities in eight-year-olds: A longitudinal study. Develop Psych, in press.

Byrne GD, Abbott KM, Thrasher S, Suomi SJ. Reorganization of dominance rank among adult males in a captive group of tufted capuchins (*Cebus apella*). Lab Primate News 1996;35:1-3.

Byrne GD, Suomi SJ. Individual differences in object manipulation in a colony of tufted capuchins. J Hum Evol, in press.

Champoux M, Higley JD, Suomi SJ. Behavioral and physiological characteristics of Indian and Chinese-Indian hybrid rhesus macaque infants. Develop Psychobiol, in press.

Champoux, M, Kriete MF, Eckhaus MA, Suomi SJ. Behavioral and physical concomitants of congenital hydrocephalus in a rhesus macaque infant. Lab Animal Sci, in press.

Champoux M, Kriete MF, Higley JD, Suomi SJ. CBC and serum chemistry differences between Indian-derived and Chinese-Indian hybrid rhesus monkey infants. Amer J Primatol 1996;39:79-84.

Clarke AS, Boinski S. Temperament and reactivity in primates. Am J Primatol 1995;37:103-126.

Damast AM, Tamis-LeMonda CS, Bornstein MH. Mother-child play: Sequential interactions and the relation between maternal beliefs and behaviors. Child Develop 1996;

Dawud-Noursi S, Sternberg KJ, Lamb ME. Domestic violence in the family context. In: Lewis M, Feiring C eds. Families, risk, and competence. Mahwah, NJ: Erlbaum, in press.

Del Carmen R, Huffman LC. Epilogue: Bridging the gap between research on attachment and psychopathology. J Consult Clin Psychol 1996;64:291-294.

Doudet D, Hommer D, Higley JD, Andreason PJ, Moneman R, Suomi SJ, Linnoila M. Cerebral glucose metabolism, CSF 5-HIAA, and aggressive behavior in rhesus monkeys. Am J Psychiat 1995;152:1782-

1787.

Fracasso MP, Lamb ME, Schölmerich A, Leyendecker B. The ecology of mother-infant interaction in Euro-American and immigrant Central American families living in the United States. Internat J Behav Develop, in press.

Genevro JL, Andreassen CJ, Bornstein MH. Young children's understanding of routine medical care and strategies for coping with stressful medical experiences. In: Bornstein MH, Genevro JL, eds. Child Development and Behavioral Pediatrics. Mahwah, NJ: Lawrence Erlbaum Associates, 1996;59-83.

Higley JD, King ST, Hasert MF, Champoux M, Suomi SJ, Linnoila M. Stability of interindividual differences in serotonin function and its relationship to severe aggression and competent social behavior in rhesus macaque females. Neuropsychopharm 1996;14:67-76.

Higley JD, Mehlman PT, Poland RE, Faucher I, Taub DM, Vickers J, Suomi SJ, Linnoila M. Correlations of CSF 5-HIAA and CSF testosterone with different types of aggressive behaviors in a nonhuman primate model. Biol Psychiat, in press.

Higley JD, Mehlman PT, Taub DM, Higley S, Fernald B, VIckers J, Lindell SG, Suomi SJ, Linnoila M. Excessive mortality in young free-ranging primates with low CSF 5-HIAA concentrations. Arch Gen Psychiatr 1996;53:537-543.

Higley JD, Suomi SJ. Reactivity and social competence affect individual differences to severe stress in children: Investigations using nonhuman primates. In: Pfeffer CR, ed. Intense stress and mental disturbance in children. New York: American Psychiatric Press, 1996;3-58.

Higley JD, Suomi SJ, Linnoila, M. A nonhuman primate model of Type II excessive alcohol consumption? (Part 1): Low CSF 5-HIAA concentrations and diminished social competence correlate with excessive alcohol consumption. Alcoholism: Clin Exp Res 1996;20:629-642.

Higley JD, Suomi SJ, Linnoila M. A nonhuman model of Type II alcoholism? (Part 2): Diminished social competence and excessive aggression correlates with low CSF 5-HIAA concentrations. Alcoholism: Clin Exp Res 1996;20:643-650.

Higley JD, Suomi SJ, Linnoila M. Progress toward developing a nonhuman primate model of alcohol abuse and alcoholism. Soc Sci Med, in press.

Horowitz SW, Lamb ME, Esplin PW, Boychuk TD, Krispin O, Reiter-Lavery L. Reliability of crieria-based content analysis of child witness statements. Legal and Criminolgical Psychology, in press.

Horowitz SW, Lamb ME, Esplin PW, Boychuk TD, Reiter-Lavery L, Krispin O. Establishing ground truth in studies of child sexual abuse. Expert Evidence, 1995;4:42-51.

Huffman LC, Del Carmen R, Bryan YE, Pedersen FA. Cry acoustics and temperament. In: Lester BM, Newman JD, Pedersen FA, eds. Biological and social aspects of infant crying. New York: Plenum Press, in press.

Hwang CP, Lamb ME, Sigel I. eds, Images of childhood. Mahwah, NJ: Lawrence Erlbaum Associates, 1996.

Jorgensen MJ, Suomi SJ, Hopkins WD. Using a computerized testing system to investigate the pre-conceptual self in primates and humans. In: Rochet P ed. The self in early infancy: Theory and research. Amsterdam: North Holland-Elsevier Press, 1995:243-256.

Lamb ME. Paternal influences on child development. In: van Dongen MCP, Frinking GAB, Jacobs MJG eds, Changing fatherhood: An interdisciplinary perspective. Amsterdam, The Netherlands: Thesis Publishers, 1995;145-157.

Lamb ME. Effects of nonparental child care on child development: An update. Canad J Psychiatr 1996:41:330-342.

Lamb ME. Review of "Fatherless America: Confronting our most urgent social problem." J Marr Fam 1996;58:526-527.

Lamb ME. Review of "Divergent realities: The emotional lives of mothers, fathers and adolescents." Soc Service Rev 1996;70:489-490

Lamb ME. Nonparental child care: Context, quality, correlates, and consequences. In: Sigel I & Renninger KA vol. eds., Child psychology in practice, Damon W series ed., Handbook of child psychology (5 e). New York: Wiley, in press.

Lamb ME. Review of "The book of David: How preserving families can cost children's lives". J Marr Family, in press. [Book Review]

Lamb ME. The role of the father in child development: An introduction and overview. In: Lamb ME ed, The role of the father in child development (3 e). New York: Wiley, in press.

Lamb ME. ed, The role of the father in child development (3 e). New York: Wiley, in press.

Lamb ME. L'influence du pere sur le developpement de l'enfant. [Paternal influences on child development]. Enfance, in press [French].

Lamb ME. The development of father-infant relationships. In: Lamb ME ed, The role of the father in child development (3 e). New York: Wiley, in press.

Lamb ME. Review of "Family, justice, and delinquency." Fam Rel, in press.

Lamb ME. Fathering in America: New challenges and champions. Contemp Psychol, in press.

Lamb ME, Billings LL. Fathers of children with special needs. In Lamb ME ed, The role of the father in child development (3 e). New York: Wiley, in press.

Lamb ME, Fracasso MP. Les dimensions der temperament: Physiologie, comportement et perceptions maternelles [The dimensions of temperament: Physiology, behavior, and maternal perceptions]. In Tessier R, Tarabulsy GM eds, Enfance et famille: Contortes de developpements [Child and family: Contexts for development] (French). Quebec City: Les presses de l'Université Laval, in press.

Lamb ME, Hershkowitz I, Sternberg KJ, Esplin PW, Hovav M, Manor T, Yudilevitch L. Effects of investigative utterance types on Israeli children's responses. Internat J Behav Develop, in press.

Lamb ME, Hwang CP. Images of childhood: An introduction. In: Hwang CP, Lamb ME, Sigel I eds, Images of childhood. Mahwah, NJ: Lawrence Erlbaum Associates, 1996;1-12.

Lamb ME, Leyendecker BR, Schölmerich A. Everyday experiences of infants in Euro-American and Central American families. In Lewis M, Feiring C eds, Families, risk, and competence. Mahwah, NJ: Erlbaum, in press.

Lamb ME, Nash A, Teti DM, Bornstein MH. Infancy. In: Lewis M, ed. Child and Adolescent Psychiatry: A Comprehensive Textbook, 2e. Baltimore: Williams & Wilkins, 1996;241-70.

Lamb ME, Sternberg KJ, Esplin PW. Making children into competent witnesses: Reactions to the amicus brief in re Michaels. Psychol, Public Policy, Law 1995;1:438-449.

Lamb ME, Wessels H. Tagesbetreuung [Daycare]. In: Keller H ed., Handbuch der kleinkindforschung (2 Auflage) [Handbook of child study (2 e)]. Bern: Huber.

Leyendecker B. Umweltwahrnehmung [Perceiving the environment]. In Keller H ed, Handbuch der Kleinkindforschung [Handbook of infant development], 2. e. Bern: Huber, in press.

Leyendecker B. Entwicklung im soziokulturellen Kontext [The sociocultural context of development]. In Keller H ed, Handbuch der Kleinkindforschung, 2. e. Bern: Huber, in press.

Leyendecker B, Lamb ME, Fracasso MP, Schölmerich A, Larson C. Playful interaction and the antecedents of attachment: A longitudinal study of Central American and Euro-American mothers and infants. Merrill-Palmer Quart, in press.

Leyendecker B, Lamb ME, Schölmerich A, & Miranda Fricke D. Contexts as moderators of observed interactions: A study of Costa Rican mothers and infants from differing socio-economic backgrounds. Inter J Behav Devel, in press.

Malkova L, Mishkin M, Suomi SJ. C comparison of early vs. late medical temporal lobe lesions on socioemotional behavior in rhesus monkeys. Annal NY Acad Sci, in press.

Mayes LC, Bornstein MH. Developmental dilemmas for cocaine-abusing parents and their children. In: Lewis M, Bendersky M. eds. Mothers, Babies, and Cocaine: The Role of Toxins in Development. Hillsdale, NJ: Lawrence Erlbaum Associates, Publishers, 1995;251-72.

Mayes LC, Bornstein MH. Infant information-processing performance and maternal education. Early Develop Parent 1995;4:91-6.

Mayes LC, Bornstein MH. Attention regulation in infants born at risk: Prematurity and prenatal cocaine exposure. In: Burack J, Enns JT, eds. Attention, Development, and Psychopathology. New York: Guilford Press, 1996;xx-xx

Mayes LC, Bornstein MH. The context of development for young children from cocaine-abusing families. In: Kato P, Mann T. eds. Health Psychology of Special Populations. New York: Plenum Press, 1996;xx-xx.

Mayes LM, Bornstein MH. The development of children exposed prenatally to cocaine. In: Luthar S, Burak J, Cicchetti D, Weisz J. eds. Developmental Psychopathology: Perspectives on Risk and Disorder. New York: Cambridge University Press, 1996;xx-xx.

Mayes LC, Bornstein MH, Chawarska K, Granger RH. Information processing and developmental assessments in three-month-old infants exposed prenatally to cocaine. Pediatrics 1995;*95*:539-545.

Mayes LC, Bornstein MH, Chawarska K, Haynes OM, Granger RH. Impaired regulation of arousal in 3-month-old infants exposed prenatally to cocaine and other drugs. Develop Psychopath 1996; 8:29-42.

Mayes LC, Feldman R, Haynes OM, Bornstein MH, Granger RH, Schottenfeld, R. Interactions between cocaine-abusing mothers and their 3- and 6-month-old infants. Inf Behav Develop,

Mehlman PT, Higley JD, Faucher I, Lilly AA, Taub DM, Vickers J, Suomi SJ, Linnoila M. CSF 5-HIAA concentration are correlated with sociality and the timing of emigration in free-ranging primates. Am J Psychiat 1995;152:907-913.

Moody KM, Adler, NT. The role of the uterus and cervix in systemic oxytocin-PGE_2 facilitated lordosis behavior. Hormones Behav 1995;29:571-580.

Newman JD, Bachevalier J. Neonatal ablations of the amygdala and inferior temporal cortex in rhesus macauqes alter vocal response to social separation during the first year of life. Brain Res, in press.

Newman JD, Bernhards DE. The affilitative vocal subsystem of squirrel monkeys. Ann NY Acad Sci, in press.

Newman JD, Farley MJ. An ethologically based, stimulus and gender-sensitive nonhuman primate model for anxiety. Prog Neuropsychopharm Biol Psychiat 1995;19:677-685.

Nsamenang AB & Lamb ME. The force of beliefs: How the parental values of the Nso of Northwest Cameroon shape children's progress toward adult models. J Appl Develop Psychol 1995;16:629-643.

Orbach Y, Lamb ME, Sternberg KJ, Williams JMG, Dawud-Noursi S. The effect of domestic violence on children's retrieval of autobiographical memory. In: Williams LM, Banyard VL eds. Trauma and memory. Thousand Oaks, CA: Sage Publications, in press.

Pascual L, Haynes OM, Galperín CZ, Bornstein MH. Psychosocial determinants of whether and how much new mothers work: a study in the United States and Argentina. Journal of Cross-Cultural Psychology 1995; 26:314-30.

Pascual L, Schulthess L, Galperín CZ de, Bornstein MH. Las ideas de las madres sobre la crianza de los hijos en Argentina [Ideas of mothers about child development in Argentina]. Revista Interamericana de Psicologia/Interamerican Journal of Psychology 1995;29:23-38.

Paul A, Keuster J, Timme A, Arnemann J. The association between rank, mating effort, and reproductive success in male Barbary macaques (*Macaca sylvanus*). Primates 1995;34:491-502.

Pedersen FA, Huffman LC, Del Carmen R, Bryan YE. Prenatal maternal reactivity predicts postnatal marriage appraisal and perceptions of infant temperament. Child Devel, in press.

Posada G, Wu-Fang Gao Y, Haaland W, Sinevaag B, Schölmerich A. Q-sort descriptions of real and ideal children by mothers and experts. In Waters E, Vaughn B, Posada G, Kondo-Ikemura K eds, Caregiving, cultural, and cognitive perspectives on secure-base behavior and working models: New growing points of attachment theory and research. Mono Soc Res Child Development, 1995;60: Serial number 244.

Rasmussen KLR, Timme A, Suomi SJ. Comparison of physiological measures of Cayo Santiago rhesus monkey females with and between social groups. Primate rep, in press.

Roberts KP, Blades M. Children's discrimination of memories for actual and pretend actions in a hiding task. Brit J Develop Psychol 1995;13:321-334.

Roberts KP, Blades M. Do children confuse memories of TV programmes and real life in their testimony? Forensic Update 1995;41:18-22.

Roberts KP, Blades M. Children's eyewitness testimony for real-life and fantasy events. Investigative and Forensic Decision-Making. Leicester, UK, British Psychological Society, in press.

Sameroff AJ, Suomi SJ. Primates and persons: A comparative developmental understanding of social organization. In: Cairns RB, Elder GH, Costello EJ, McGuire A eds. Development science. New York: Plenum Press, in press.

Sams-Dodd F, Newman JD. Effects of administration regime on the psychomimetic properties of d-amphetamine in squirrel monkey (*Saimiri sciureus*). Pharm, Biochem, Behav, in press.

Schölmerich A, Lamb ME, Leyendecker B, Fracasso MP. Mother-infant interactions and attachment security in Euro-American and Central-American immigrant families. Inf Behav Devel, in press

Schrader L, Hammerschmidt K. Computer-aided analysis of acoustic parameters in animal vocalisations: A multi-parmetric approach. Bioacoustics, in press.

Slater AM, Brown E, Mattock A, Bornstein MH. Continuity and change in habituation in the first four months from birth. J Reprod Infant Psychol 1996;14:187-94.

Sternberg, KJ. Fathers, the missing parents in research on family violence. In: Lamb ME ed. The role of the father in child development (3rd ed.). New York: Wiley, in press.

Sternberg KJ, Dawud-Noursi SD. Effects of domestic violence on children's behavior problems: Multiple perspectives. In: Tessier R, Tarabulsy GM eds., Child and Family: Contexts for development. Quebec City, Quebec, QU: Les presses de l'Universite Laval, in press.

Sternberg KJ, Lamb ME, Dawud-Noursi S. Using multiple informants and cross-cultural research to study the effects of domestic violence on developmental psychopathology: Illustrations from research in Israel. In: Luthar SS, Burack JA, Cicchetti D, Weisz, J eds. Developmental psychopathology: Perspectives on risk and disorder. New York: Cambridge University Press, in press.

Sternberg KJ, Lamb ME, Dawud-Noursi S. Understanding domestic violence and its effects: Making sense of divergent reports and perspectives. In: Holden GW, Geffner R, Jouriles EW eds. Children exposed to family violence. Washington, DC: American Psychological Association, in press.

Sternberg KJ, Lamb ME, Hershkowitz I. Child victims and witnesses in Israel: Evaluating innovative practices. In: Bottoms BL, Goodman GS eds., International perspectives on child witnesses. Thousand Oaks, CA: Sage Publications, 1996;62-76.

Sternberg KJ, Lamb ME, & Hershkowitz I. Child sexual abuse investigations in Israel. Crim Just Behav, 1996;23:322-337.

Sternberg KJ, Lamb ME, Hershkowitz I, Esplin PW, Redlich A, Sunshine N. The relationship between investigative utterance types and the informativeness of child witnesses. J App Develop Psychol, in press.

Suomi SJ. Influence of Bowlby's Attachment Theory on research on nonhuman primate biobehavioral development. In: Goldberg S, Muir R, Kerr J, eds. Attachment theory: Social, developmental, and clinical perspectives. Hillsdale NJ: The analytic Press, 1995;185-201.

Suomi SJ. Effects of differential social experience on biological and behavioral development in rhesus monkeys. Europ Neuropsychopharmacol, in press.

Suomi SJ. Long-term effects of early experiences on social, emotional, and physiological development in nonhuman primates. In: Keshevan MS, Murra RM, eds. Neurodevelopmental models of adult psychopathology. Cambridge: Cambridge University Press, in press.

Suomi SJ. Biological, maternal, and life-style interactions with the psychosocial environment: Primate models. In: Hertzman C, Kelly S, Bobak M, eds. Environmental and nonenvironmental determinants of the East-West life expectancy gap in Europe. Amsterdam: Kluver Press, in press.

Suomi SJ. Nonverbal communication in nonhuman primates: Implications for the emergence of culture. In: Molnar P, Segerstrale U eds. Where nature meets culture: Nonverbal communication in social interactions. Hillsdale NJ: Lawrence Erlbaum, in press.

Suomi, SJ, Levine S. Psychobiology of integenerational effects of trauma: Evidence from animal studies. In: Daniele Y, ed. International handbook of multigenerational legacies of trauma. New York: Plenum Press, in press.

Suomi SJ, Novak MA, Well A. Aging in rhesus monkeys: Different windows on behavioral continuity and change. Develop Psychol, in press.

Tamis-LeMonda CS, Bornstein MH. Variation in children's exploratory, nonsymbolic, and symbolic play: An explanatory multidimensional framework. In: Rovee-Collier CR, ed. Advances in Infancy Research, vol 10. Norwood, NJ: Ablex, 1996;37-78.

Timme A. Gender differences in establishment of mother-infant bonds in a semifree-ranging group of Barbary macaques (*Macaca sylvanus*). In: Roeder JJ, Thierry B, Anderson JR, Herrenschmidt N eds. Current primatology (Vol II). Strausbourg: Universite Louis Pasteur, 1996:273-278.

Timme A. Sex differences in infant integration in a semi-free ranging group of Barbary macaques (Macaca sylvanus) at Salem, FRG. Am J Primatol 1995;37:221-231.

Venuti P, Bornstein MH, Toniatti C, Rossi G. Analisi funzionale del linguaggio materno rivolto a babini di 13 mesi. Giornale Italiano di Psicologia 1996;

Venuti P, Rossi G, Spagnoletti MS, Bornstein MH. Gioco simbolico e non simbolico a 20 mesi: comportamenti di gioco del babino e della madre. Età Evolutiva 1996;

Volling BL, MacKinnon-Lewis C, Rabiner D, Baradaran LP. Children's social competence and sociometric status: Further exploration of aggression, social withdrawal, and peer rejection. Develop Psychopath, in press.

von Eye A, Kreppner K, Spiel C, Weßels H. Life events spacing and order in individual development. In Kindermann TA, Valsiner J eds Construction of self and social relationships: Combinatorial research in developmental psychology. Hillsdale, NJ: Erlbaum, 1995: 147-164.

Weßels H. Betreuerverhalten und seine Bestimmungsfaktoren in Tageseinrichtungen für Kinder unter drei Jahren [Child care and regulation in daily care for children under three]. In: Tietze W (ed) Neue Entwicklungen in der Kleinkindpädagogik [New developments in early childhool education]. Berlin: Luchterhand, in press.

Wessels H, Lamb ME, Hwang CP. Cause and causality in daycare research: An investigation of group differences in Swedish child care. Europ J Educ Psychol 1996;11:231-245.

Westergaard GC, Green JA, Muhin-Hauser C, Suomi SJ. The use of naturally occurring copper and iron tools by monkeys. Hum Evol, in press.

Westergaard GC, Suomi SJ. The manufacture and use of bamboo tools by monkeys: Possible implications for the development of material culture among east Asian hominids. J Archeolog Sci 1995;22:677-681.

Westergaard GC, Suomi SJ. Mirror inspection varies with age and tool-using ability in tufted capuchin monkeys (Cebus apella). Hum Evol 1995;10:217-223.

Westergaard GC, Suomi SJ. The stone tools of capuchins (Cebus apella). Int J Primatol 1995;16:1017-1024.

Westergaard GC, Suomi SJ. Hand preference for stone artifact production and tool-use by monkeys: Possible implications for the evolution of right-handedness in hominids. J Hum Evol 1995;29:2-8.

Westergaard GC, Suomi, SJ. Lateral bias for rotational behavior in tufted capuchin monkeys (*Cebus apella*). J Comp Psychol 1996;110:199-202.

Westergaard GC, Suomi SJ. Stone-throwing by capuchins (*Cebus apella*): A nonhuman primate model of Homo hailis throwing habits. Folia Primatolog, in press.

Westergaard GC, Suomi SJ. Hand preferences for a bimanual task in tufted capuchins and rhesus macaques (*Macaca mulatta*). J Comp Psychol, in press.

Westergaard GC, Suomi SJ. Lateral bias in capuchin monkeys (*Cebus apella*): Concordance between parent-offspring dyads. Develop Psychobiol, in press.

Project Numbers - Annual Report 1996

LABORATORY OF COMPARATIVE ETHOLOGY (LCE)

ZO1 HD 01106-13	Developmental Continuity of Individual Differences in Reactivity in Monkeys Stephen J. Suomi, Ph.D.
ZO1 HD 01107-13	Adaptation of Laboratory Reared Monkeys to Field Environments Stephen J. Suomi, Ph.D.
ZO1 HD 01112-10	Effects of Home and Out-of-Home Care on Child Development Michael E. Lamb, Ph.D.
ZO1 HD 01114-09	Individual Differences in Physical and Affective Functioning in Infancy Michael E. Lamb, Ph.D.
ZO1 HD 01115-09	Effects of Domestic Violence and Evaluation of Children's Testimony Michael E. Lamb, Ph.D.
ZO1 HD 01116-09	Social Development Across the Lifespan in Diverse Cultures and Ecologies Michael E. Lamb, Ph.D.
ZO1 HD 01117-09	Behavioral Pediatrics: Coping with Medical Experiences and Development under Cocaine Exposure Marc H. Bornstein, Ph.D.
ZO1 HD 01119-09	Specificity of Mother-Infant Interaction Marc H. Bornstein, Ph.D.
ZO1 HD 01120-09	Parenting and Infancy Activity: A Multiculture Perspective Marc H. Bornstein, Ph.D.
ZO1 HD 01122-09	Longitudinal Assessment of Children's Mental and Social Abilities Marc H. Bornstein, Ph.D.
ZO1 HD 01123-06	Physiological Correlates and Neural Mechanisms of the Infant Cry and Related Vocalizations John Newman, Ph.D.
Z01 HD 01124-06	Genetic and Experiential Influences on the Development of Primate Vocal Behavior John Newman, Ph.D.

ZO1 HD 01126-1199

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 01106-13 LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Developmental Continuity of Individual Differences in Reactivity in Monkeys

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

S. J. Suomi	Head	LCE, NICHD
M. Champoux	Staff Fellow	LCE, NICHD
G. D. Byrne	IRTA Fellow	LCE, NICHD
D. Smucny	Pre-IRTA Fellow	LCE, NICHD
W. Airosa	Pre-IRTA Fellow	LCE, NICHD
S. Lindell	Pre-IRTA Fellow	LCE, NICHD
C. Shannon	Pre-IRTA Fellow	LCE, NICHD
A. Van Rooy	Tech IRTA Fellow	LCE, NICHD

COOPERATING UNITS *(if any)*

NIAAA (Higley, Weld, Linnoila, Goldman); NIMH (Doudet); LABS (Mehlman, Taub); U of Puerto Rico (Phoebus); U of California (Boyce); U of Minnesota (Gunnar)

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Comparative Behavioral Genetics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.5	2.5	5.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project investigates primate biobehavioral development through comparative longitudinal studies of rhesus and capuchin monkeys, with special emphasis on characterizing individual patterns of differential behavioral and physiological responses to environmental novelty and challenge and on determining long-term developmental consequences for individuals of different genetic backgrounds reared in different physical and social environments. Research carried out during FY96 (a) replicated and extended previous findings regarding early experience effects on the development of circadian rhythmicity in glucocorticoid release in rhesus monkey infants; (b) demonstrated the efficacy of surrogate-peer rearing procedures for the development of species-normative patterns of behavior and physiology in nursery-reared rhesus monkey infants and juveniles; (c) successfully cross-fostered rhesus monkey infants selectively bred for extreme (either high or low) CSF levels of 5-HIAA (the primary central serotonin metabolite) to foster mothers of differing behavioral and physiological propensities for long-term longitudinal study; (d) demonstrated a significant relationship between serotonin metabolism rates and sleep-wakefulness patterns in group-living rhesus monkey juveniles and adults; (e) documented systematic seasonal variation in serotonin metabolism in free-ranging rhesus monkey adolescent and adult males; (f) characterized changes in heartrate and heartrate variability associated with reconciliation behavior following aggressive exchanges in rhesus monkey adult females; and (g) characterized patterns of adrenocortical response to brief separation in infant and juvenile capuchin monkeys and demonstrated a predictive relationship between magnitude of cortisol response and subsequent propensity for object manipulation and proto-tool use behavior in this primate species.

Project Description:

Objectives:

This project has five basic objectives: (1) to characterize individual differences among rhesus monkeys in their biobehavioral reactions to mild environmental challenges and to assess the long-term developmental continuity of such differences under standardized rearing conditions; (2) to determine and evaluate genetic and experiential factors that either alone or in concert influence the expression of such individual differences throughout development; (3) to describe the relationships among behavioral measures of relative reactivity and a variety of physiological concomitants, including patterns of hypothalamic-pituitary-adrenal activation, autonomic nervous system reactivity, monoamine metabolism, and immune system responsiveness; (4) to develop intervention strategies for altering ontogenic trajectories of individuals identified in early infancy as being at high risk for subsequent development of extreme reactions to species-normative environmental challenges; and (5) to assess the generality of specific findings from laboratory-born rhesus monkeys not only to wild populations of rhesus monkeys but also to capuchin monkeys (Cebus apella) and other primate species.

Methods Employed:

Rhesus monkey infants of known parentage are studied longitudinally in settings in which selected aspects of their physical and social environment are varied in systematic fashion. Behavioral and physiological data encompassing multiple levels of analysis are routinely gathered from each subject at various points under predetermined environmental conditions from birth to senescence. These data include frequent time-sampled focal animal behavioral observations utilizing sophisticated category-based scoring systems, neurological and neonatal reflex tests administered during infancy, periodic sampling of various hormones, neurotransmitters, and their metabolites in saliva, blood, and CSF under carefully controlled environmental conditions, and systematic telemetric monitoring of psychophysiological activity in different physical and social settings. Some subjects receive occasional PET, SPEC, and MRI brain scans under isoflurane restraint. Subsets of these measures are also being obtained in prospective longitudinal developmental studies of capuchin monkeys.

Major Findings:

Several studies completed this past year examined the effects of specific early experiences on behavioral and physiological development in rhesus monkey infants and juveniles. Repeated sampling of saliva in nursery-reared infants from birth onward documented the emergence of the prototypical primate pattern of circadian rhymicity in cortisol output by 3 weeks of age, consistent with extant human findings (adjusted for species differences in maturational rate). Remarkably, by 6 weeks of age this initially robust circadian rhythm began to deteriorate in the nursery-reared rhesus infants. Moreover, by 2-3 months of age, cortisol output appeared to be closely linked to specific activities within the nursery environment, including some that did not directly involve the infants themselves; such linkage had the effect of disrupting the normative circadian pattern of cortisol secretion. However, in succeeding months the adrenocortical response to these routine nursery activities was significantly blunted and a more definitive circadian pattern of cortisol secretion re-emerged. Comparable developmental data regarding circadian rhymicity and sensitivity of adrenocortical response to routine events have been collected from mother-reared infants (and from their mothers as well); those data are currently being analyzed.

A second set of studies compared and contrasted the behavioral and physiological consequences of two standardized nursery rearing procedures for rhesus monkey infants and juveniles: continuous access to like-reared agemates (peer-only rearing) vs. "only" 2 hours of access to peers 5 days per week, the rest of the time spent individually housed with inanimate surrogate "mothers" (surrogate-peer rearing), beginning at 30 days and continued through 6 months of age. Somewhat surprisingly, surrogate-peer reared monkeys exhibited significantly higher levels of locomotion and social play, and lower levels of infantile social clinging and self-directed behavior, than their peer-only reared counterparts; in this respect, surrogate-peer reared monkeys more closely resembled mother-reared infants growing up in naturalistic settings than did peer-only reared subjects. Moreover, surrogate-peer reared monkeys exhibited less extreme adrenocortical responses to standardized environmental challenges at 3, 4, and 5 months of age than peer-only reared subjects (and, indeed, even lower than mother-reared monkeys of comparable ages). While these findings, suggesting that "less is better" from the standpoint of exposure to peers, might seem counterintuitive, they are consistent with attachment theory principles regarding separate, often incompatible, socialization roles for maternal-like and peer relationships. Monoamine metabolite samples from these differentially reared monkeys are currently under analyses. Additional behavioral, adrenocortical, and monoamine data will be collected throughout adolescence and into adulthood.

A third study investigating the effects of differential early rearing experiences involved foster-rearing rhesus monkey neonates selectively bred for extreme (high vs. low) patterns of central monoamine metabolism (as assessed by CSF concentrations of 5-HIAA) with foster mothers who differed systematically in their relative sociality and impulsiveness, respectively. Given previous findings linking low CSF 5-HIAA levels with asocial, impulsive, and often destructively aggressive behavior in juvenile, adolescent, and adult monkeys, this cross-fostering design will enable substantial disentanglement of heritable and experiential influences; more importantly, it will facilitate identification and characterization of interactions among these various influences, which are typically dynamic and complex. Data collection for this prospective longitudinal cross-fostering study is currently underway, encompassing a variety of behavioral and physiological measures sampled repeatedly from the infants, their foster mothers, and their biological mothers.

Three other studies investigated relationships between individual differences in 5-HIAA levels and certain other aspects of rhesus monkey life. In the first, a significant negative relationship was found between CSF 5-HIAA concentrations and both overall activity levels and sleep latencies in rhesus monkey families living in indoor-outdoor pens. A second study documented seasonal variability in 5-HIAA levels in free-ranging juvenile, adolescent, and adult rhesus monkey males. CSF 5-HIAA concentrations were highest during autumn months, rising significantly from summer levels. For these wild-living rhesus monkeys, the autumn months encompass the annual breeding season, and perhaps not surprisingly, these seasonal changes in 5-HIAA levels were most pronounced in the adult males and least pronounced in the juveniles.

The third area of 5-HIAA-focused research investigated the potential efficacy of enhancing 5-HIAA levels (and possibly reducing impulsive and aggressive behavior) via dietary supplements of tryptophane, a serotonin precursor. Such efforts to enhance 5-HIAA levels, as well as reduce antisocial behavior, have not been generally effective to date in studies involving group-living monkeys. In contrast, a study completed this past year involving individually housed adult male monkeys, some of whom exhibited occasional episodes of self-injurious behavior (SIB), clearly demonstrated not only significant increases in CSF 5-HIAA levels, but also significant "dose-dependent" decreases in SIB, concomitant with tryptophane dietary supplements. Interestingly, these tryptophane-associated increases in CSF 5-HIAA were observed only in subjects who previously had exhibited SIB; non-SIB monkeys failed to show this increase when given the same tryptophane dietary supplements, even though their pre-tryptophane 5-HIAA concentrations were statistically indistinguishable from those of the monkeys exhibiting SIB. These results suggest that risk for developing SIB may be associated with unusually labile patterns of serotonin metabolism.

Another long-term study in which data collection was completed this past year examined neurochemical and psychophysiological concomitants of reconciliation behavior following aggressive exchanges among group-living rhesus monkey females. Whereas aggressive behavior was characterized by sharp increases in heartrate, reconciliation (i.e., affiliation with former opponents immediately after aggressive episodes) was associated with significant decreases in heartrate, relative to preconflict, conflict, and nonconflict matched control periods. Behavioral and neurochemical data from this study are still being analyzed.

Finally, long-term study of capuchin monkey biobehavior development yielded several interesting findings regarding both species normative patterns and individual differences in response to environmental challenge. First, short-term (2 hr.) separations from familiar conspecifics produced adrenocortical responses in 6-month and 1-year-old capuchin monkeys that were basically intermediate in magnitude between species-normative separation-induced cortisol levels for rhesus monkeys and other Old World monkey species, on the one hand, and the extreme levels characteristic of squirrel monkeys, a New World monkey species like capuchins, on the other. However, baseline cortisol levels of these capuchin subjects more closely resembled Old World monkey norms, both of which were significantly lower than squirrel monkey baseline cortisol levels. Second, individual differences in adrenocortical response to separation were highly stable from 6 to 12 months of age, paralleling a wealth of data from rhesus monkeys and other macaque species. Third, these differences in adrenocortical response were associated with differences in activity state patterns recorded in the first 3 months of life. Finally, individual differences in cortisol levels during separation were themselves predictive of subsequent object manipulation and proto-tool using behavior during the second year of life, which, in turn, appear to be predictive of marked individual differences in complex tool-using propensities later in life (see Project #Z01 HD 01107-13 LCE). These data represent the first concrete identification of any factor that is predictive of the dramatic individual differences in tool-using abilities that characterize this unusual primate species.

Significance to Biomedical Research and the Program of the Institute:

Delineating and understanding the ways in which genetic and environmental factors interact to shape the course of an individual's ontogeny represents perhaps the most basic challenge in the study of developmental processes in whole organisms. The significance of the research encompassed by this project lies in its focus on both genetic and environmental sources of variance, its use of multiple longitudinal measures of both behavioral and physiological processes representing several levels of analysis, and its emphasis on early detection of extreme cases and subsequent strategies for intervention, using advanced nonhuman primate subjects who share many aspects of early behavioral and physiological development with human infants but who grow to maturity in only a fraction of the time required for comparable human development.

Proposed Course of Project:
In the coming year we plan to continue our longitudinal studies of rhesus monkey infants selectively bred for high vs. low CSF 5-HIAA levels and reared in different social environments, as well as initiating a new longitudinal study of infants selectively bred for extremes of both 5-HIAA functioning and adrenocortical reactivity and subsequently reared differentially. In addition, the long-term developmental consequences of differential early experience with environmental controllability will be studied prospectively in a new cohort of selectively bred and differentially reared rhesus monkey infants. Studies of the interrelationships of various behavioral measures and physiological indices will continue, including direct experimental manipulations of subsets of these variables under carefully controlled laboratory conditions, as well as generalization studies in free-ranging populations of rhesus monkeys living in the wild. We also plan to examine patterns of covariance between serotonin metabolism and blood cholesterol levels in rhesus monkey of different ages and rearing background, as well as study the effects of supplementing nursery-reared infants' diets with free fatty acids (present in mothers' milk but lacking in standard formula diets). Finally we will continue our prospective longitudinal investigations of biobehavioral development and response to environmental novelty and challenge in the LCE's capuchin monkey colony.

Publications:

Boyce WT, Higley JD, Jenerin J, Champoux M, Suomi SJ. Tympanic temperature asymmetry and stress behavior in rhesus monkeys and children. Arch Ped Adol Med, in press.

Byrne GD, Abbott KM, Thrasher S, Suomi SJ. Reorganization of dominance rank among adult males in a captive group of tufted capuchins (*Cebus apella*). Lab Primate News 1996;35:1-3.

Byrne GD, Suomi SJ. Individual differences in object manipulation in a colony of tufted capuchins. J Hum Evol, in press.

Champoux M, Higley JD, Suomi SJ. Behavioral and physiological characteristics of Indian and Chinese-Indian hybrid rhesus macaque infants. Develop Psychobiol, in press.

Champoux, M, Kriete MF, Eckhaus MA, Suomi SJ. Behavioral and physical concomitants of congenital hydrocephalus in a rhesus macaque infant. Lab Animal Sci, in press.

Champoux M, Kriete MF, Higley JD, Suomi SJ. CBC and serum chemistry differences between Indian-derived and Chinese-Indian hybrid rhesus monkey infants. Amer J Primatol 1996;39:79-84.

Del Carmen R, Huffman LC. Epilogue: Bridging the gap between research on attachment and psychopathology. J Consult Clin Psychol 1996;64:291-294.

Doudet D, Hommer D, Higley JD, Andreason PJ, Moneman R, Suomi SJ, Linnoila M. Cerebral glucose metabolism, CSF 5-HIAA, and aggressive behavior in rhesus monkeys. Am J Psychiat 1995;152:1782-1787.

Higley JD, King ST, Hasert MF, Champoux M, Suomi SJ, Linnoila M. Stability of interindividual differences in serotonin function and its relationship to severe aggression and competent social behavior in rhesus macaque females. Neuropsychopharm 1996;14:67-76.

Higley JD, Mehlman PT, Taub DM, Higley S, Fernald B, VIckers J, Lindell SG, Suomi SJ, Linnoila M. Excessive mortality in young free-ranging primates with low CSF 5-HIAA concentrations. Arch Gen Psychiatr 1996;53:537-543.

Higley JD, Mehlman PT, Poland RE, Faucher I, Taub DM, Vickers J, Suomi SJ, Linnoila M. Correlations of CSF 5-HIAA and CSF testosterone with different types of aggressive behaviors in a nonhuman primate model. Biol Psychiat, in press.

Higley JD, Suomi SJ. Reactivity and social competence affect individual differences to severe stress in children: Investigations using nonhuman primates. In: Pfeffer CR, ed. Intense stress and mental disturbance in children. New York: American Psychiatric Press, 1996;3-58.

Higley JD, Suomi SJ, Linnoila, M. A nonhuman primate model of Type II excessive alcohol consumption? (Part 1): Low CSF 5-HIAA concentrations and diminished social competence correlate with excessive alcohol consumption. Alcoholism: Clin Exp Res 1996;20:629-642.

Higley JD, Suomi SJ, Linnoila M. A nonhuman model of Type II alcoholism? (Part 2): Diminished social competence and excessive aggression correlates with low CSF 5-HIAA concentrations. Alcoholism: Clin Exp Res 1996;20:643-650.

Higley JD, Suomi SJ, Linnoila M. Progress toward developing a nonhuman primate model of alcohol abuse and alcoholism. Soc Sci Med, in press.

Huffman LC, Del Carmen R, Bryan YE, Pedersen FA. Cry acoustics and temperament. In: Lester BM, Newman JD, Pedersen FA, eds. Biological and social aspects of infant crying. New York: Plenum Press, in press.

Malkova L, Mishkin M, Suomi SJ. C comparison of early vs. late medical temporal lobe lesions on socioemotional behavior in rhesus monkeys. Annal NY Acad Sci, in press.

Mehlman PT, Higley JD, Faucher I, Lilly AA, Taub DM, Vickers J, Suomi SJ, Linnoila M. CSF 5-HIAA concentration are correlated with sociality and the timing of emigration in free-ranging primates. Am J Psychiat 1995;152:907-913.

Pedersen FA, Huffman LC, Del Carmen R, Bryan YE. Prenatal maternal reactivity predicts postnatal marriage appraisal and perceptions of infant temperament. Child Devel, in press.

Suomi SJ. Influence of Bowlby's Attachment Theory on research on nonhuman primate biobehavioral development. In: Goldberg S, Muir R, Kerr J, eds. Attachment theory: Social, developmental, and clinical perspectives. Hillsdale NJ: The analytic Press, 1995;185-201.

Suomi SJ. Biological, maternal, and life-style interactions with the psychosocial environment: Primate models. In: Hertzman C, Kelly S, Bobak M, eds. Environmental and nonenvironmental determinants of the East-West life expectancy gap in Europe. Amsterdam: Kluver Press, in press.

Suomi SJ. Effects of differential social experience on biological and behavioral development in rhesus monkeys. Europ Neuropsychopharmacol, in press.

Suomi SJ. Long-term effects of early experiences on social, emotional, and physiological development in nonhuman primates. In: Keshevan MS, Murra RM, eds. Neurodevelopmental models of adult psychopathology. Cambridge: Cambridge University Press, in press.

Suomi, SJ, Levine S. Psychobiology of integenerational effects of trauma: Evidence from animal studies. In: Daniele Y, ed. International handbook of multigenerational legacies of trauma. New York: Plenum Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01107-13 LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Adaptation of Laboratory Reared Monkeys to Field Environments

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

S. J. Suomi	Head	LCE, NICHD
A. Timme	Fogarty Fellow	LCE, NICHD
G. Westergaard	IRTA Fellow	LCE, NICHD
P. O. Wagner	Research Psychologist	LCE, NICHD
C. Price	Biologist	LCE, NICHD
K. L. Rasmussen	Senior Staff Fellow	LCE, NICHD
A. Lauter	Tech IRTA	LCE, NICHD

COOPERATING UNITS *(if any)*

Caribbean Primate Research Center (Berard); New England Regional Primate Center (Jorgensen); SUNY-Buffalo (Berman); U Colorado (Laudenslager), U Florida (Boinski); U Massachusetts (Novak); U Wisconsin (Kalin); Istituto di Psicologia, CNR (Visalberghi)

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Comparative Behavioral Genetics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.8	3.5	2.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project investigates how rhesus monkeys and other nonhuman primate species born and raised under different laboratory conditions adapt to placement into environments that contain specific physical and social features of the monkeys' natural habitat. Adaptation is assessed by examining behavioral repertoires and by monitoring a variety of physiological systems in these subjects, yielding broad-based indices of relative physical and psychological well-being. The responses of subjects to experimental manipulations of selected features of their respective environments are also assessed in similar fashion. Whenever, possible field data are collected for appropriate comparisons. An additional focus is on investigating the cognitive, behavioral, and social processes involved in adaptation to new settings or circumstances. Capuchin monkeys are employed in many of these studies because they are unique among monkey species in their propensity to manufacture and use tools to modify their physical environment.

Research carried out during FY96 (a) documented species-normative age, gender, dominance, and seasonal influences on behavioral repertoires and adrenocortical responsiveness among the rhesus monkeys maintained in the LCE's 5-acre outdoor enclosure following completion of a new shelter facility; (b) identified developmentally stable individual differences in behavioral, psychophysiological, and adrenocortical reactions to environmental challenges in wild-living rhesus monkey infants and juveniles; (c) demonstrated significant relationships between heartrate and adrenocortical responsiveness and specific aspects of maternal behavior in wild-living rhesus monkey females; (d) developed computer-based procedures for testing memory capabilities of captive rhesus monkeys; (e) characterized developmental, postural, and task-specific correlates of handedness in capuchin monkeys; and (f) expanded the known range of complex tool-using behaviors in captive capuchin monkeys and documented the sharing of both food and tools between small social groups housed in adjacent pens.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

The basic objectives of this long-term project are: (1) to study the process behavioral and physiological processes involved in adaptation to naturalistic physical environments in rhesus monkeys born and reared under laboratory conditions, (2) to determine the extent to which these observed adaptive processes generalize to rhesus monkeys with different rearing backgrounds and current home environments, as well as to other primate species, (3) to assess the effects of experimental manipulations of selected features of the monkeys' captive environment in order to simulate specific phenomena characteristic of their natural habitats, and (4) to utilize these data to develop laboratory environments that are explicitly designed to foster various aspects of psychological well-being for their respective primate inhabitants, as well as to provide a means for assessing their capabilities for cognitive, behavioral, and social adaptation.

Methods Employed:

A major component of this project centers on longitudinal study of a group of 23-year-old rhesus monkeys and 4 generations of their offspring, all of whom live year-round in a 5-acre outdoor enclosure with shelter on the grounds of the NIHAC. The group-founding 23-year-old monkeys were born and raised under laboratory conditions without exposure to any conspecifics besides themselves; the same is true of their offspring. A comparison group of rhesus monkeys born and reared under comparable conditions in the same laboratory as the founding study group subjects, but whose members have spent the intervening years living in indoor pens at the University of Massachusetts, Amherst, is involved in parallel studies and data collection protocols, as are other long-term social groups of rhesus monkeys, some living in indoor-outdoor laboratory pens and some living in the field.

Time-sampled observations utilizing sophisticated behavioral scoring systems provide the primary mode of data collection for monkeys living in the 5-acre field enclosure at the NIHAC, and similar data are available from the various comparison groups. Monkeys in these groups are each observed for 10-minute periods of focal animal data collection several times each week by specially trained LCE personnel. During each observation period the frequency, duration, and sequence of the focal subject's activities are recorded according to a exhaustive category scoring system; the partners in each social interchange involving the focal subject are also identified, as is the nature and sequence of the partners' behavior during the interaction. Additionally, the location of all other group members is recorded at periodic intervals throughout each focal animal's observation period. The resulting data set is then entered into the basic longitudinal data base that has been maintained on each group member since birth. Appropriate files from this data base can be utilized to address specific empirical questions concerning longitudinal behavioral development and adaptations within and between the respective study groups. Additionally, these monkeys' patterns of environmental adaptation can be compared with behavioral and physiological patterns of conspecifics having different backgrounds and different current living environments, as well as with those of other primate species. In addition, new techniques for telemetric collection of psychophysiological data from the rhesus monkeys living in free-ranging environments have been developed and formal data collection continued this past year. A variety of techniques for using foraging patterns to assess memory functions and resource utilization under standardized conditions are also being tested in different settings.

Finally, because capuchin monkeys are unique among monkey species in their propensity to use tools to modify their physical environment, systematic investigations of object manipulation and the development of tool use are carried out in some of the capuchin groups. In addition, tests of sophisticated cognitive and perceptual-motor capabilities are conducted within the capuchin monkeys' home environments. In these tests the monkeys first learn to operate a joystick that moves a cursor on a video display. Software has been developed to present subjects with a variety of tasks tapping different perceptual-motor and cognitive capabilities that can be "solved" via appropriate movement of the cursor; solutions to individual trials are rewarded via delivery of a food pellet. This apparatus is also being utilized in comparative studies with rhesus monkeys and chimpanzees.

Major Findings:

Construction of a mjaor new shelter facility at the LCE's 5-acre outdoor field station was completed at the start of FY96, ending a 2-year period of disruption of routine data collection on the rhesus monkey troop housed within the 5-acre enclosure. Subsequent behavioral observations of this troop revealed that the monkeys quickly returned to the pre-construction behavioral repertoires and hierarchical pattern of social group organization. These repertoires included species-normative age, gender, and rank differences in activity patterns and habitat utilization, seasonal variation in time budgets, and normative patterns of parental care, infant development, and gender-specific behavioral and social status changes associated with the onset of puberty. Detailed comparisons of these post-construction patterns with behavioral and physiological data collected prior to and during the construction period are currently underway; these analyses focus on evaluating the degree to which individual behavior repertoires were disrupted by the construction process and their relative rate of return to pre-construction patterns as a function of age, gender, social status, and individual temperament.

Spatial memory capabilities of the rhesus monkeys in the LCE's field station troop were compared with those of rhesus monkeys living in two different indoor laboratory settings; the comparisons focused on the monkey's ability to recognize novel objects placed within a complex stimulus grid, as well as to detect changes in the location of familiar objects within the same grid. Field station monkeys showed spatial memory capabilities comparable to those living in the more restricted indoor settings when given extensive daily exposure to the stimulus grids. However, when exposure to the stimulus grid was limited to 2 hours per day, interesting group differences emerged: field monkeys were more responsive to changes in location of familiar objects than were monkeys in the indoor groups, whereas the reverse was true for identifying novel objects. Age and gender differences in exploratory patterns appeared similar across the different monkey groups.

Analysis of behavioral and physiological data from a long-term prospective longitudinal study of individual differences in developmental trajectories within a cohort of rhesus monkey infants growing up in wild troops on Cayo Santiago island (PR) focused this past year on reactions to brief maternal separations associated with mating activities and to the birth of younger siblings. In general, the reactions to brief maternal separation were considerably milder during the second year of life than during the first, although individual differences in the relative severity of behavioral disruption were relatively stable across years. The birth of a sibling was associated with sharp decreases in time spent in contact with or near the mother and concomitant increases in passivity, but not in active distress behavior; these changes tended to be greater in male than in female juveniles. Analysis of morphometric and psychophysiological data collected from these juveniles during their annual capture for veterinary examination revealed stable gender and individual differences in weight, skinfold, heartrate patterns, and vocalizations during heartrate recording periods. These data represent the first demonstration of significant developmental continuities of individual differences in behavior and physiology during the first two years of life in rhesus monkeys growing up in the wild.

Another study involving the wild rhesus monkeys living on Cayo Santiago island focused on the relationship between individual differences in behavioral and physiological response to brief capture (for veterinary examination) and patterns of maternal behavior exhibited by multiparous adult females. Females exhibiting high and stable heartrates also tended to have relatively high plasma cortisol levels following capture, replicating previous field data collected from infants and juveniles, as well as adolescent males. These "high-reactive" females exhibited lower amounts of ventral-ventral and dorsal-ventral contact, and higher rates of initiated contact breaks with their infants than did "low-reactive" females; they also tended to groom other adult females more, and had their infants contact their nipples less often and take greater responsibility for maintaining proximity, than their low-reactive counterparts. These data provide the first empirical evidence that maternal behavior patterns are influenced by maternal temperament in wild-living groups of rhesus monkeys.

A different line of research carried out this past year in collaboration with researchers at the New England Regional Primate Research Center continued studies of primate cognitive capabilities using an automated video-game based testing apparatus, in which monkeys are trained to move a cursor on a video screen via movement of a hand-drive joystick, then presneted with tasks that can be solved via specific cursor movements. Two new memory tasks were developed and pilot-tested with rhesus monkey subjects. The first task utilized a delayed matching-to-sample paradigm; the second task involved tests of spatial memory following different standardized delay periods.

Extensive research investigating the tool-using capabilities of capuchin monkeys was continued in FY96, expanding considerably the known range and complexity of tool-using tasks the species is able to master under captive living conditions, including the use of tools to facilitate capture of live prey (ants). In addition, a paradigm was developed that enabled groups of capuchins housed in adjacent pens to obtain and share highly desirable food items only if tools to obtain the food were also shared. Several monkeys in both groups mastered this complex tool-and-food-sharing task, providing the first evidence of tool-sharing in any other primate species besides humans and chimpanzees. The degree to which laterality (handedness) was involved in the various tool-using taks was also investigated in several studies. In general, a greater right-handed bias was found in adult subjects than in juveniles, and this right-handed bias was significantly stronger for tasks requiring a bipedal posture than those that could be solved while sitting or maintaining a quadrapedal posture.

Finally, data from several field studies investigating the relationship between social structure, environmental context, and specific vocal patterns in several New World primate species were published in FY96.

Significance to Biomedical Research and the Program of the Institute:

The discovery that laboratory-born-and-reared rhesus monkeys readily adapt to naturalistic habitats and retain species-normative behavioral repertoires and patterns of social organization in the absence of any exposure to wild-born, ferally experienced adult conspecifics is of considerable significance in its demonstration that such complex behavioral capabilities and characteristics are clearly preserved in the genetic heritage of members of an advanced nonhuman primate species, even after many generations of laboratory living. Additionally, the relative ease, speed and thoroughness of species-normative adaptation to a naturalistic environment shown by these rhesus monkeys should encourage primate conservation efforts based on the establishment of captive breeding colonies with subsequent introduction into protected naturalistic habitats. They also provide a basis for establishing scientifically rigorous standards regarding the creation of laboratory environments that "promote the psychological well-being" for captive primate subjects, as has recently been mandated by statute through an amendment to the Animal Welfare Act. The demonstration of sophisticated tool-using capabilities in capuchin monkeys provides evidence for continuity between these monkeys and apes and humans in several important dimensions and serves as a basis for focused investigation of cognitive processes and neurobehavioral correlates that underlie the use of tools in these diverse species. Finally, the development of procedures that promote or enhance well-being in nonhuman primate subjects growing up in captive environments can provide insights regarding aspects of physical and social environments that serve to promote or enhance well-being in human infants, children, and the elderly.

Proposed Course:

We plan to continue detailed longitudinal observations of the rhesus monkey troop living in the 5-acre outdoor enclosure at the NIHAC and on the comparison group living indoors at the University of Massachusetts. We also plan to continue longitudinal data collection on the free-ranging rhesus monkey troops living on Cayo Santiago. Video-based cognitive testing will be expanded to include additional tasks and a wider range of primate subjects. Additional studies of complex tool use in capuchin monkeys will investigate the basis for the dramatic individual differences in tool-using capabilities within the LCE's capuchin colony, as well as identifying neurobiological correlates that might underlie such differences.

Publications:

Berman CM, Rasmussen KLR, Suomi SJ. Group size, infant development, and social networkds: A natural experiment with free-ranging rhesus monkeys. Animal Behav, in press.

Boinski S. Stress responses in primates: Proximate mechanisms in the evolution of social organization. In: Foster SA, Endler JA eds. Geographic variation in behavior: An evolutionary perspective. Oxford: Oxford University Press, in press.

Boinski S, Campbell AF. Use of trill vocalizations to coordinate troop movement among white-faced capuchins: A second field test. Behaviour, in press.

Clarke AS, Boinski S. Temperament and reactivity in primates. Am J Primatol 1995;37:103-126.

Jorgensen MJ, Suomi SJ, Hopkins WD. Using a computerized testing system to investigate the pre-conceptual self in primates and humans. In: Rochet P ed. The self in early infancy: Theory and research. Amsterdam: North Holland-Elsevier Press, 1995:243-256.

Paul A, Keuster J, Timme A, Arnemann J. The association between rank, mating effort, and reproductive success in male Barbary macaques (*Macaca sylvanus*). Primates 1995;34:491-502.

Rasmussen KLR, Timme A, Suomi SJ. Comparison of physiological measures of Cayo Santiago rhesus monkey females with and between social groups. Primate rep, in press.

Samerooff AJ, Suomi SJ. Primates and persons: A comparative developmental understanding of social organization. In: Cairns RB, Elder GH, Costello EJ, McGuire A eds. Development science. New York: Plenum Press, in press.

Suomi SJ. Nonverbal communication in nonhuman primates: Implications for the emergence of culture. In: Molnar P, Segerstrale U eds. Where nature meets culture: Nonverbal communication in social interactions. Hillsdale NJ: Lawrence Erlbaum, in press.

Suomi SJ, Novak MA, Well A. Aging in rhesus monkeys: Different windows on behavioral continuity and change. Develop Psychol, in press.

Timme A. Sex differences in infant integration in a semi-free ranging group of Barbary macaques (Macaca sylvanus) at Salem, FRG. Am J Primatol 1995;37:221-231.

Timme A. Gender differences in establishment of mother-infant bonds in a semifree-ranging group of Barbary macaques (*Macaca sylvanus*). In: Roeder JJ, Thierry B, Anderson JR, Herrenschmidt N eds. Current primatology (Vol II). Strausbourg: Universite Louis Pasteur, 1996:273-278.

Westergaard GC, Green JA, Muhin-Hauser C, Suomi SJ. The use of naturally occurring copper and iron tools by monkeys. Hum Evol, in press.

Westergaard GC, Suomi SJ. The manufacture and use of bamboo tools by monkeys: Possible implications for the development of material culture among east Asian hominids. J Archeolog Sci 1995;22:677-681.

Westergaard GC, Suomi SJ. Mirror inspection varies with age and tool-using ability in tufted capuchin monkeys (Cebus apella). Hum Evol 1995;10:217-223.

Westergaard GC, Suomi SJ. The stone tools of capuchins (Cebus apella). Int J Primatol 1995;16:1017-1024.

Westergaard GC, Suomi SJ. Hand preference for stone artifact production and tool-use by monkeys: Possible implications for the evolution of right-handedness in hominids. J Hum Evol 1995;29:2-8.

Westergaard GC, Suomi, SJ. Lateral bias for rotational behavior in tufted capuchin monkeys (*Cebus apella*). J Comp Psychol 1996;110:199-202.

Westergaard GC, Suomi SJ. Stone-throwing by capuchins (*Cebus apella*): A nonhuman primate model of Homo hailis throwing habits. Folia Primatolog, in press.

Westergaard GC, Suomi SJ. Hand preferences for a bimanual task in tufted capuchins and rhesus macaques (*Macaca mulatta*). J Comp Psychol, in press.

Westergaard GC, Suomi SJ. Lateral bias in capuchin monkeys (*Cebus apella*): Concordance between parent-offspring dyads. Develop Psychobiol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD01112-10LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Home and Out-of-Home Care on Child Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. E. Lamb	Head, SSED	LCE, NICHD
Other:	H. Weßels	Visiting Fellow	LCE, NICHD
	K.J. Sternberg	Research Psychologist	LCE, NICHD

COOPERATING UNITS *(if any)*

Institute of Psychology, University of Goteborg (Broberg, Hwang); Institute for Applied Socialization Research, Berlin (L. Ahnert); S. Viernickel.

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Section on Social and Emotional Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves a longitudinal study conducted in Sweden examining the effects of center day care, family day care, and home care on the development of 145 children recruited in 1982 at an average of 16 months of age. Initial multivariate analyses indicated that prior to the start of formal schooling, type of care had no reliable impact on child development, but that the quality of home care and the quality of alternative care had substantial effects on the children's verbal abilities, their social skills, and their behavioral attitudes. Subsequent analyses of data gathered shortly after the children started school indicated that the development of verbal and mathematical abilities, as well as the development of personal maturity (as measured by field-independence, ego-control, and ego-resilience), were affected by the varying child-care histories the children experienced since their first enrollment in the study. These analyses indicated that family daycare has modest but significant negative effects on both verbal abilities and personal maturity, whereas center-based care has beneficial effects in both domains. The effects of the quality of home and alternative care appeared to diminish as the children moved into the formal educational system. Reassessment of these children at 14-15 years of age began in late FY96. In this phase, data are being obtained by interviewing the children and their parents, as well as from official school records.

In a related study, SSED staff are assessing the psychophysiological and behavioral tendencies of young infants at home in order to assess the effects of individual differences in emotional reactivity and infant-mother attachment on the adaptation to out-of-home care. Data collection has now been completed, and data reduction and analysis are under way.

Project Description:

Objectives:

Since the establishment of the SSED in July 1987, staff have been involved in the analysis of data from a longitudinal study in Sweden examining the effects of center daycare, family daycare, and home care on social, personality, and intellectual development. Analyses have been designed to explore the origins and temporal course of individual differences among the children, using novel analytic techniques that have been developed for such multi-measure longitudinal studies.

Guided by previous research in the USA, staff expected that type of care (home care, center daycare, or family daycare) would have no significant impact on aspects of child development, but that the quality of home and out-of-home care, the child's temperament, and his/her initial patterns of sociability would predict individual differences in later indices of social and intellectual competence.

Recently, SSED staff and collaborators undertook a longitudinal study of the physiological and behavioral correlates and predictors of adaptations to out-of-home care in infancy. In this study, 60 infants in Berlin are being assessed immediately prior to and after enrollment in day care centers. The principal focus is on the effects of individual differences in reactivity and infant-mother attachment on behavioral sigin of distress and adaptation to new careproviders. In this study, 12- to 18-month-old infants and their mothers were observed at home before day care began. The Strange Situation procedure was used to assess the security of infant-mother attachment, and psychophysiological baseline assessments were obtained. Psychophysiological (heartrate reactivity and cortisol) were assessed in each of the first days in day care, including those when mothers were present and those on which they left. Videotaped records were obtained to allow synchronization of psychophysiological and behavioral sequences. Data collection has now been completed and data reduction and analysis are under way.

Methods Employed:

145 children on the waiting lists for childcare facilities in Sweden were enrolled in the Swedish Longitudinal Study at an average of 16 months of age. In an initial visit to the homes, data were collected about family background, social networks, care arrangements, relative parental involvement, temperament, and the child's initial response to the visiting adult. In a second visit, the HOME inventory (Caldwell, 1970) and the Belsky and Walker (1980) observation checklist were employed to assess the quality of the home environment. Children enrolled to center-based daycare or family daycare entered these settings within two weeks of this first assessment. Six weeks later their childcare facilities were visited by a member of the research staff to obtain observational and interview data about the care arrangement and its quality.

One and two years thereafter the families were visited again. During these visits, stranger sociability was assessed, and the parents were questioned about their care arrangements. In a second visit, the quality of the home environment, paternal involvement in child care, and the children's personal maturity and peer play skills were measured. Trained psychologists administered the Griffiths Developmental Scales in order to assess the verbal abilities of children in both phases. Again, for the children enrolled in family or center-based daycare, these facilities were visited to collect data about the quality of the care facility and the children's behavior in the alternative care setting. The fourth and fifth waves of data collection took place prior to enrollment in elementary school and when the children were in second grade. During these visits, the children's verbal and mathematical abilities were tested by trained assistants, and data about paternal involvement, the children's personal maturity, peer play skills, and current care arrangements were gathered. Additionally, data about the quality of the pre-school and alternative care settings were collected again. In phase five, an intensive interview with the elementary school teacher was conducted in order to obtain additional information about the children's peer behavior, adjustment to school, cognitive and social skills, and personality.

In the new study, children's heartrate and salivary cortisol levels are being recorded at home, as well as on several occasions following enrollment in day care. Responses to separation are also being observed and coded. The Strange Situation procedure is being used to assess the security of infant-mother attachment both before day care commences and 6 weeks after enrollment. Behavioral and physiological responses are recorded on each of the days mothers remain with their infants in the day care center, as well as on the days on which the infants are truly left by their mothers. The behavioral indices tap the use of both careproviders for emotional support and soothing. Videotaped records permit synchronization of the behavioral and psychophysiological records.

Major Findings:

One of the most important goals of child care research has been to determine whether or not nonparental care has adverse effects on child development. Answering this question involves making casual attributions about the origins of differences between groups of children who have experienced divergent nonparental care arrangements. Because children and families are not randomly assigned to child care trajectories, researchers face problems when trying to demonstrate causal relationships. One goal of the Goteborg Child Care Study has been to show that the discovery of significant differences between groups must initiate extensive efforts to validate and interpret the findings, avoiding the misinterpretation of group differences that are actually attributable to preselection effects of confounding variables. By rigorously identifying such confounds, researchers can accumulate support for hypothesized causal relationships, but they cannot 'prove' them because unknown and unmeasured confounding variables may always have important effects.

Parental ratings of field independence, ego-resilience, and ego-control obtained using the California Q-set when the children averaged 28, 40, 80, and 101 months of age revealed virtually no difference between the developmental trajectories of the children in home care and in center-based daycare. By contrast, ego-undercontrol decreased less, while ego-resilience and field independence increased less, in children who attended family daycare than in the children in either center care or exclusive parental care. The quality of home and out-of-home care, as well as socioeconomic status and family background, did not qualify these effects of the type of care. Other analyses of the same data were designed to evaluate the Five Factor Model of personality. The factors identified by John et al. in a study of adolescents were barely reliable when the children averaged 29 months of age, but became increasingly reliable in the assessments which took place when the children averaged 40, 80, and 101 months of age. Results of a cross-validation, employing data obtained when the children were in second grade, indicated that these factors were meaningfully related to measures of the childrens' cognitive abilities and school adjustment.

The divergent developmental trajectories of the children in the home-care and the center-based daycare groups differed with respect to the development of verbal abilities as well. When the children were eight years old and in the second grade of elementary school, tests and teachers' ratings of children's cognitive abilities were administered to the 123 children (65 girls) remaining in the study. Tested ability was related to the number of months children had spent in center-based day care before 3 1/2 years of age. Structural and dynamic aspects of the child care quality also predicted cognitive abilities among those children who had spent at least 36 months of their preschool years in out-of-home care. Both tested and rated cognitive abilities in second grade were related to earlier measures of verbal ability and to paternal involvement during the pre-school years.

No data have yet been analyzed from the Berlin Longitudinal Study.

Significance for Biomedical Research and the Program of the Institute:

This program of research has underscored the quality of out-of-home care and the role of factors outside the care setting, such as the quality of home care and maternal characteristics, when evaluating the effects of daycare. Our research also highlights the difficulties inherent in attempts to measure children's behavioral development, and it underscores the value of longitudinal studies of adjustment and problem behaviors in different child-rearing settings. More generally, this research provides information about the factors that account for the emergence, maintenance, and alteration of individual differences among children. Such findings provide valuable basic evidence about developmental processes, while informing public policy concerning issues such as daycare and parental leave policies. Research on individual differences in patterns of adaptation promises to help better predict how different children will be affected by enrollment in day care.

Proposed Course of Project:

Ongoing analyses and data collection are designed to further explore longitudinal associations in several other domains, including the development of peer and social skills and gender-relevant aspects of self-perception. These children and their parents are being reassessed in the 9th grades at school. In the Berlin study, all data collection has now been completed. Data reduction and analysis will be central in FY97.

Publications:

Broberg AG, Wessels H, Lamb ME, & Hwang CP. The effects of day care on the development of cognitive abilities in eight-year-olds: A longitudinal study. Develop Psych, in press.

Lamb ME. Effects of nonparental child care on child development: An update. Canad J Psychiatr 1996:41:330-342.

Lamb ME. Nonparental child care: Context, quality, correlates, and consequences. In: Sigel I & Renninger KA vol. eds., Child psychology in practice, Damon W series ed., Handbook of child psychology (5 e). New York: Wiley, in press.

Lamb ME, Wessels H. Tagesbetreuung [Daycare]. In: Keller H ed., Handbuch der kleinkindforschung (2 Auflage) [Handbook of child study (2 e)]. Bern: Huber.

Weßels H. Betreuerverhalten und seine Bestimmungsfaktoren in Tageseinrichtungen für Kinder unter drei Jahren [Child care and regulation in daily care for children under three]. In: Tietze W (ed) Neue Entwicklungen in der Kleinkindpädagogik [New developments in early childhool education]. Berlin: Luchterhand, in press.

Wessels H, Lamb ME, Hwang CP. Cause and causality in daycare research: An investigation of group differences in Swedish child care. Europ J Educ Psychol 1996;11:231-245.

von Eye A, Kreppner K, Spiel C, Weßels H. Life events spacing and order in individual development. In Kindermann TA, Valsiner J eds Construction of self and social relationships: Combinatorial research in developmental psychology. Hillsdale, NJ: Erlbaum, 1995: 147-164.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01114-09 LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Individual Differences in Physical and Affective Functioning in Infancy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. E. Lamb	Head, SSED	LCE, NICHD
Other:	K. Sternberg	Research Psychologist	LCE, NICHD
	B. Leyendecker	Fogarty Fellow	LCE, NICHD

COOPERATING UNITS *(if any)* Dept. of Psych., U of Osnabruck, Germany (Keller); Inst. for Applied Socialization Research, Berlin, Germany (Ahnert); Dept. of Psych., U of Halle, Germany (Schölmerich); Dept. of Anthropology, Washington State U (Hewlett); Dept. of Human Development, U of Maryland (Porges); Inst. of Psychological Research, U of Costa Rica (Miranda); Université Laval (Tessier); Baradaran; Larson; Viernickel.

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Section on Social and Emotional Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project focuses on long-term consequences of individual differences in physical, social, and affective functioning and the description of early interactions in diverse ecological contexts. In one study, we studied infant-mother interaction and attachment in infants from upper-middle class Euro-Americans and lower-class Central American families, developing culturally-sensitive criteria for evaluating normative social behavior and development. Regardless of social or ethnic background, mothers spent comparable amounts of time in discrete activities such as feeding, caring for, and playing with their infants. Systematic analyses demonstrated that the quality of social interaction within dyads changes dramatically depending on the functional and social context. As a result, extended observations are necessary to obtain reliable indices of individual differences. Comparable observations of mothers and infants in Costa Rica, Quebec, Germany, the Central African Republic, and Colombia are designed to further explore the effects of culture and context on early interactions.

Project Description:

Objectives:

Most students of behavioral development now agree that variations in developmental processes and outcomes are jointly determined by complex interactions between inherited dispositions and tendencies, on the one hand, and by experiential histories on the other. Empirically demonstrating such patterns of influence has proven difficult, however, because it demands the ability to conduct longitudinal studies incorporating sensitive measures of both biogenetic factors, developmental ecologies, and early interaction. One goal of the SSED staff has been to demonstrate these patterns of influence by undertaking detailed observational research on infants.

In one such multicultural investigation, SSED staff are developing techniques to describe the interaction between mothers and their infants in early life and to assess the effects of particular patterns of parenting, infant and maternal characteristics, and family patterns on subsequent attachment behavior on infants from divergent (and under-studied) backgrounds, using both upper-middle class Euro-American (EA), lower-class Central American (CA) immigrants; and groups of families in Costa Rica. It is believed that individual differences in the quality of prior interaction between mothers and children determine infants' attachments to their mothers, and that the quality of attachment is a powerful predictor of the infant's psychosocial adjustment and behavior in the future. These associations have only been demonstrated rigorously in middle-class Caucasian populations, however. Little is known about patterns of infant-mother interaction in different ethnic and cultural subgroups, and it is not known whether similar predictive associations exist in non-mainstream populations. One of the component studies involves repeated observations of mother-child interaction to permit assessment of the influence of parenting styles on attachment and socioemotional development. Other studies have focused on the effects of functional and social context on infant experience and patterns of infant-adult interaction.

Methods:

For the study of Euro- and Central American mothers and infants in the Washington metropolitan area, mothers were contacted before their first-born infants were 15 weeks old. Four home visits took place when the children were three months of age, and other visits were made when the children were 4, 8 and 12 months old. When the infants were three months old, 4-hour observations of parent-infant interaction and infant behavior were scheduled throughout the day in overlapping units to ensure coverage of a 12-hour cycle. The four 3-month assessments had the same general format, incorporating age-appropriate measures for assessing maternal perceptions of infant temperament, along with measures of parental stress, social support, life stress, and marital relationships. The 4-, 8-, and 12-month assessments included observations of structured interactions such as feeding and teaching, as well as age-appropriate measures of infant temperament, including both maternal questionnaires and behavioral observations. The final 12-month assessment included laboratory observations of infant-mother attachment and social interaction.

Observational data were also collected in Costa Rica and are being gathered in Germany, Quebec, Colombia, and the Central African Republic. These samples were selected to represent equal proportions of subjects from at least two subsamples in each country. When the infants were three months, they were observed throughout the day in overlapping units to ensure coverage of 12-hour cycles, as was true in the USA.

Major Findings:

In our studies of the Euro-American and Central-American mothers in the Washington area, mother-infant interaction during the free play, teaching, and feeding situations at 4, 8, and 12 months of age were coded microanalytically. Individual differences on the discrete measures of infant behavior and behavioral coordination during bouts of mother-infant play were not stable; however, they were related to Strange Situation behavior at some but not all points in time. Frequency measures of maternal behavior were not related to the infants' attachment status, but the responsivity scores were stable over time and strongly related to the quality of attachment. In both groups, mothers of secure infants scored around the middle, whereas mothers of resistant infants scored at either higher or intermediate levels. Mothers of avoidant infants tended to score lower in the Euro-American sample and higher in the Central American sample. Disorganized infants in both groups were less involved than secure infants in the tactile exploration of objects at all three ages. Central American mothers of disorganized infants consistently scored higher, whereas the Euro-American mothers of disorganized infants scored either lower or higher on the measures of responsivity. Didactic maternal behavior in the teaching situation decreased as the infants grew older, whereas task-oriented infant behavior increased with age. Individual differences in maternal teaching behavior were not stable over time, although the level of maternal vocalization was stable. Infants and mothers in the two samples differed with respect to discrete behaviors, but there were only minor differences between the samples with respect to both levels of mutual coordination and the probability of secure attachment behavior in the Strange Situation. Frequency and duration measures of maternal and infant behavior in the teaching sessions were largely unrelated to later attachment status, but individual differences in indices of mutual coordination were significantly related to attachment security. Dyads who were later classified as disorganized had negative scores on a measure of joint attention to objects. Resistant dyads were characterized by high levels of coordination of social attention, while disorganized dyads had very low or negative coordination scores. Mutual coordination of maternal teaching was highest in dyads involving securely attached infants. Analysis of behavior in the feeding sessions has yet to be completed.

Initial comparative analyses of data focused on the general patterning of everyday experiences in families comprising recent immigrants from Central America and in families comprising well-educated Euro-Americans. Descriptive analyses revealed remarkable similarities and few differences in the everyday experiences of infants in these two diverse groups. Both groups of mothers spent most of their time playing with, feeding, caring for, or holding their infants. Fathers spent little time with their infants during the day and their presence did not affect the amount of time spent in various social contexts, although significantly more holding occurred when the fathers were present. Other people spent more time with the infants in the Central American families and less time alone with the infants in the Euro-American families than fathers did. Mother and infant vocalizations and mutual attention occurred more frequently during play than in bouts of feeding or holding.

Subsequent analyses were focused on the influence of context on the observed levels of dyadic interaction and on the influence of length of observation on the reliability of interactional measures. There were no group differences in the amounts of time spent by the mothers and infants in well-attuned states, or in different functional contexts, but the EA dyads spent more time in disharmonious states. Securely attached dyads in both samples spent more time in well-attuned states, as well as in playful interactions, rather than not interacting. Forty-five minute blocks of observation yielded highly unreliable measures of individual differences, but reliability increased considerably as the duration of the observations was expanded. The amounts of time spent in well-attuned and in disharmonious interactions varied substantially across contexts, with notable differences between the two groups. The results indicate that subcultural differences can be maximized by focussing on single contexts and minimized by averaging across a variety of naturally occurring contexts.

Mothers' reports of their daily activities when the infants in the Washington study were 8 months and 12 months of age were also analyzed. Detailed descriptions of the previous 24 hours were obtained by interviewing the mothers and compiling recall diaries. Results to date indicated that the infants' experiences and activities were very similar in both groups, and the effects of the mothers', fathers', or others' presence on ongoing activities were similar, too. The groups differed with regard to (1) the circadian distribution of activities, (2) opportunities for interactions with various people, and (3) the differences between weekday and weekends. Overall, the social worlds of the Central American children were characterized by the simultaneous presence of several people and thus by multiple social partners. The social worlds of the Euro-American children were characterized by more opportunities for dyadic interactions and by exposure to fewer partners.

We then investigated the relationship between mothers' long-term socialization goals and their evaluations of infants' behavior in a variety of everyday contexts, including feeding, sleeping, playing, and diaper changing. In terms of their long-term socialization goals, we found that the Central American mothers stressed a desire for their children to learn proper demeanor and respect for authority, whereas the Euro-American mothers stressed the importance of fostering autonomy and independence. Examination of mothers' evaluation of infant behavior indicated that the Central American mothers placed greater weight on the appropriateness of the infants' behavior in each of these contexts, whereas the Euro-American mothers focused more on external factors such as the presence of others, location, and timing. These findings suggest that the extent to which infants are considered responsible partners in the smooth functioning of everyday activities varies culturally and relates coherently to culture-specific emphases on interdependence or independence.

Observations of 20 three-month-old Costa Rican infants with middle class mothers and another 20 with working class mothers also revealed remarkable similarities and few differences in everyday experiences of infants in these two groups. Infants spent most of their waking hours being cared for or held, and engaging in visual and verbal interaction. Fathers spent little time with their infants. When their fathers were present, middle class infants were more likely to be held and not to interact with anyone, whereas the working class infants were more likely to be fed. Regardless of social class, other people spent more time alone with the infants than fathers did. Mother and infant vocalizations and mutual attention occurred more frequently during play than in bouts of caring, feeding, or holding. There were few social class differences, although working class mothers spent more time feeding their infants than middle class mothers did.

Subsequent analyses of these data were designed to (1) study the impact of length of observation and context on our measures of interactional engagement, and (2) compare the interactional experiences of the infants in the two groups in various functional (e.g., feeding, object play) and social (with mother, with mother and others) contexts. Attuned and disharmonious interactions, as well as the frequency of positive affect, soothing, and vocalization, varied considerably across the functional contexts, as they did in the US samples. In addition, disharmonious interactions increased and interactional engagement decreased when mothers and infants were joined by others. Highly unstable measures of individual differences were obtained when observations were limited to 45-minute blocks, but stability increased considerably as the duration of the observations expanded, as in the previous study. The groups did not differ with respect to amounts of time spent in various functional and social contexts, in attuned or disharmonious states, or in high levels of interactional engagement. Within some of the functional contexts, however, significant group differences in levels of attuned interactions, infant vocalization, and maternal response vocalization were found. Overall, functional and social contexts clearly moderated interactional experiences. SES effects on verbal and other interactional measures were limited to some contexts and may thus represent the infants' overall experiences quite poorly. Consequently, comparisons based on a single context may be inadequate for studies of subjects from differing socioeconomic backgrounds.

Significance for Biomedical Research and the Program of the Institute:

The significance of this research lies in its focus on infants in diverse cultural contexts. By providing detailed observations and descriptions of culturally-specific parenting behavior and infant experiences in everyday life, the results extend our knowledge of normative social behavior. Exceptions to previously established associations between mother-infant interaction and infant-mother attachment may be revealed, which in turn could have important implications for the assessment of developmental processes. Since most studies involve families already assimilated into the mainstream culture, this project may disclose ethnocentric biases in our understanding of how environmental influences affect children's socioemotional development. Research on various subcultures affords the opportunity to obtain a more inclusive understanding of biobehavioral development.

Proposed Course of Project:

Analyses of interaction during the feeding sessions are now being conducted, as are analyses of the effects of functional context (i.e., feeding, caretaking, play) on measures of interactional harmony in dyads observed in Germany, Colombia, Costa Rica, Quebec, and the Central African Republic. These data will permit further insight into the existence and importance of culturally divergent childcare practices.

Publications:

Fracasso MP, Lamb ME, Schölmerich A, Leyendecker B. The ecology of mother-infant interaction in Euro-American and immigrant Central American families living in the United States. Internat J Behav Develop, in press.

Lamb ME, Fracasso MP. Les dimensions der temperament: Physiologie, comportement et perceptions maternelles [The dimensions of temperament: Physiology, behavior, and maternal perceptions]. In Tessier R, Tarabulsy GM eds, Enfance et famille: Contortes de developpements [Child and family: Contexts for development] (French). Quebec City: Les presses de l'Université Laval, in press.

Lamb ME, Leyendecker BR, Schölmerich A. Everyday experiences of infants in Euro-American and Central American families. In Lewis M, Feiring C eds, Families, risk, and competence. Mahwah, NJ: Erlbaum, in press.

Lamb ME, Nash A, Teti DM, Bornstein MH. Infancy. In Lewis M ed Child and adolescent psychiatry: A comprehensive textbook (2 e). Baltimore: Williams and Wilkins, 1996;241-270.

Leyendecker B. Entwicklung im soziokulturellen Kontext [The sociocultural context of development]. In Keller H ed, Handbuch der Kleinkindforschung, 2. e. Bern: Huber, in press.

Leyendecker B. Umweltwahrnehmung [Perceiving the environment]. In Keller H ed, Handbuch der Kleinkindforschung [Handbook of infant development], 2. e. Bern: Huber, in press.

Leyendecker B, Lamb ME, Fracasso MP, Schölmerich A, Larson C. Playful interaction and the antecedents of attachment: A longitudinal study of Central American and Euro-American mothers and infants. Merrill-Palmer Quart, in press.

Leyendecker B, Lamb ME, Schölmerich A, & Miranda Fricke D. Contexts as moderators of observed interactions: A study of Costa Rican mothers and infants from differing socio-economic backgrounds. Inter J Behav Devel, in press.

Posada G, Wu-Fang Gao Y, Haaland W, Sinevaag B, Schölmerich A. Q-sort descriptions of real and ideal children by mothers and experts. In Waters E, Vaughn B, Posada G, Kondo-Ikemura K eds, Caregiving, cultural, and cognitive perspectives on secure-base behavior and working models: New growing points of attachment theory and research. Mono Soc Res Child Development, 1995;60: Serial number 244.

Schölmerich A, Lamb ME, Leyendecker B, Fracasso MP. Mother-infant interactions and attachment security in Euro-American and Central-American immigrant families. Inf Behav Devel, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01115-09 LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Domestic Violence and Evaluation of Children's Testimony

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	M. E. Lamb	Head, SSED	LCE, NICHD
Other:	K. J. Sternberg	Research Psychologist	LCE, NICHD
	S. Noursi	IRTA Fellow	LCE, NICHD
	Y. Orbach	IRTA Fellow	LCE, NICHD
	K. P. Roberts	Fogarty Fellow	LCE, NICHD

COOPERATING UNITS *(if any)* Hebrew U, Jerusalem (Greenbaum); Jerusalem Municipality (Lorey); Ministry of Social Welfare, Jerusalem (Eden, Horowitz, Hovav); U. of Haifa (I. Hershkowitz); U. of NC, Chapel Hill (M. Everson); U. of Cincinnati (B. Boat); Children's Hospital, San Diego (D. Chadwick; J. Landsverk); M. Breitman-MacDowell; T. Darvish; P.W. Esplin; C. Larson; L. Yudilevitch.

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Section on Social and Emotional Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

  ☒ (a1) Minors

  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This program of research involves two projects. The goal of the first project is to explore the effects of domestic violence on children who were either victims of physical abuse, witnesses of spousal abuse, both victims and witnesses, or neither victims nor witnesses. These children were first assessed in 1988/9 when they were between 8 and 12 years of age, and the children, their parents, and their teachers have since been reinterviewed, analyses of the data are now in progress. To date, analyses have focused on demonstrating and evaluating the implications of major discrepencies between reports by different informants concerning both family violence and children's adjustment.

The goal of the second project is to develop and assess techniques for enhancing the informativeness of child witnesses and for evaluating the credibility of their accounts. In one study, investigators around the United States and in Israel provided transcripts of audio- or videotaped interviews along with case files to establish case facts independent of the statements. The statements were assessed using a revision of the Criterion-Based Content Analysis (CBCA) technique, while case facts were independently rated using "ground truth" assessment scales developed by SSED staff. Accounts of events that appeared very likely to have happened indeed contained more CBCA criteria than accounts that appeared implausible, although the differences were not large. Other studies in the research program have focused on the relationship between interviewer style and the quality of information provided by young children. Several studies have confirmed that open-ended questions elicit longer and more detailed responses than more focused questions, regardless of the number of incidents experienced and the language (English or Hebrew) in which the interview was conducted. The use of anatomical dolls neither affects the association nor enhances the informativeness of young children. Recent studies have shown that interviewers can increase the length and richness of children's accounts by following SSED-designed protocols designed to probe recall memory and reduce the reliance on more focused questions which are more likely to elicit erroneous information. Currently, efforts are being made to extend and evaluate these interview protocols.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

Effects of domestic violence:

It appears self-evident that domestic violence has adverse effects on children, but there have been few methodologically sound studies of the nature, magnitude, and permanence of these effects. Before our research began, there had been no adequately controlled studies comparing the effects of witnessing and/or being the victim of domestic violence, no controlled investigations of the factors protecting some children and making others more susceptible to adverse effects, and no studies exploring children's phenomenological perceptions of domestic violence. The first project has been designed specifically to address these lacunae and to examine the extended effects of experiencing domestic violence by following the children's development longitudinally and by obtaining information about both family history and children's adjustment from a variety of informants with differing perspectives.

Children's testimony:

One objective of the research in this program is to evaluate and improve upon the Criterion-Based Content Analysis (CBCA) system for eliciting and assessing statements made by children who are alleged to have been victims or witnesses of sexual abuse. The validity of the CBCA technique is being evaluated by comparing information from investigative case files with ratings on the CBCA criteria performed by coders blind to the status and background of the cases. In addition, these and other transcripts are exhaustively coded to explore the relationship between interviewer utterance type and the richness and length of the responses elicited. These descriptive accounts have helped SSED staff to develop interview techniques which increase the length and richness of children's accounts. Field studies have been designed to assess the relative effectiveness of differing scripts employed by forensic investigators to elicit information from young alleged victims and witnesses.

Methods Employed:

Effects of domestic violence:

110 children, their parents, homeroom teachers, and peers provided information about the target children's functioning at the time of recruitment in 1988/9. The families, selected by the staff of the Ministry of Welfare in Jerusalem and Tel Aviv, Israel, were divided into four groups: 31 children who experienced chronic physical abuse by a parent, with at least one incident occurring 6 months prior to the interview; 16 who witnessed chronic spouse abuse; 29 who both witnessed and were victims of parental abuse; and 31 matched controls who experienced no domestic violence. Cross-validation of group classification was determined through interviews with parents and children.

Parents provided demographic data and described their children's temperament and behavior problems. With the assistance of a researcher, children completed instruments designed to measure their self esteem, behavior problems, depression, social support networks, perceptions of responsibility, predictability and control of domestic violence, social problem solving skills, and perceptions of their peers and parents. Teachers provided information concerning the children's academic performance and behavior. Peers provided information about their classmates using sociometric techniques. Three years after the initial phase of data collection, family caseworkers completed questionnaires focusing on changes in family circumstances and current parent-child relationships. Homeroom teachers later provided information about the children's classroom behavior, academic performance, and peer relationships. Mental health professionals and school personnel provided information about children's psychological functioning, truancy, special educational needs, physical health, delinquency, and performance on standardized tests. In FY 94 through FY 96, the children, as well as their mothers and fathers, were reinterviewed. Parents were asked to describe their children's behavioral problems, while children undertook clinical diagnostic interviews described any behavior problems, reported any symptoms of depression, and completed a variety of other measures designed to assess their level of functioning in diverse developmental domains.

Children's testimony:

In the second project, a large sample of audio- and videotaped victim/witness statements elicited by professionals are being collected from several different sites in the United States and Israel. The contents of these statements are systematically evaluated by research assistants using the Criterion Based Content Analysis (CBCA) scoring system and compared with independent ratings of forensic evidence about the case, including medical evidence, material evidence, and witness or suspect statements. The results of the statement analyses are compared with case facts to assess the validity of the children's accounts and the accuracy of the procedure. The quality of interview is also evaluated by examining the association between the types of questions posed by interviewers and the amount and quality of information provided by the children. In this research, coders focus on five types of interviewer utterance: open-ended or invitational, facilitative, directive, leading, and suggestive--as well as the length (number of words) and richness (number of details) of the children's responses. This information is also being used to explore how the quality of interview, indexed by the strategies employed to elicit information, is related to CBCA scores.

Based on the results of these studies, SSED staff developed and are field-testing interview protocols designed to increase the amount and quality of information provided by young victims of alleged abuse. In the first such study, investigators in Israel systematically alternated between a script characterized by an open-ended rapport-building style and another with a directive rapport-building style. Later, investigators in Florida systematically alternated among three protocols which continued the scripting though part of the substantive phases of the interview. In a related study, we examined the effects on interview style and children's testimony (as assessed by the richness and length of their accounts) of the use of anatomically detailed dolls. In a third study, we are systematically evaluating the length and richness of interviews in relation to the length of time that has passed since the children were allegedly abused.

Major Findings:

Effects of Domestic Violence:

Overall, the results demonstrate that domestic violence has effects on child development that vary in nature depending on the type of violence and the source of information about the child's adjustment, leading us to emphasize the necessity of including more than one informant in research on domestic violence. Considerable differences among informants are evident regarding both the effects on children and the existence/types of domestic violence itself. In spite of the fact that the behavior problem scales were standardized separately for boys and girls, the girls in this sample were found to be more adversely affected than their male counterparts.

Preliminary analyses of the children's accounts of conflicts with their parents were conducted to explore whether (a) greater reliance on over-general memory retrieval in children was related to depression, and (b) domestic violence affected the specificity of children's memory retrieval. As predicted, there was a positive correlation between children's 'generic-categoric' memory retrieval and their depression level. Children who scored higher on depression gave significantly higher proportion of 'generic-categoric' responses to questions concerning child-parent and inter-parental disagreements. However, there was no evidence that autobiographical memory was affected by domestic violence.

Children's Testimony:

Transcripts of forensic interviews with 98 alleged victims of child sexual abuse were scored for the presence or absence of the CBCA criteria believed to be more characteristic of accounts concerning experienced than nonexperienced events. As predicted, more of the CBCA criteria were present in accounts independently rated as Likely or Very Likely to have occurred than in accounts of events deemed Unlikely or Very Unlikely to have occurred. Several of the criteria were especially helpful in distinguishing between plausible and implausible accounts, but the group differences were not as dramatic as those reported in earlier studies.

In several studies, we have confirmed and replicated the prediction that open-ended invitations yielded significantly longer and more detailed responses than directive, leading, or suggestive utterances regardless of age and culture (Israel, United States). Younger children provide briefer and less detailed responses, however. Detailed psycholinguistic analyses of 45 interviews of 4- to 12-year-old children by police investigators in the United States revealed that the superiority of invitations was greater when the children reported experiencing three or more, rather than only one, incidents of abuse. Invitations were rarely used, however, and the investigators often failed to elicit more information from children who reported multiple incidents of abuse than from children who reported only one incident.

In another study, we compared 16 investigative interviews in which anatomical dolls were employed for the purposes of demonstration with another 8 in which they were not used. Cases were matched with respect to other characteristics of the children and the alleged events. Children interviewed with dolls provided an equivalent number of details and spoke as many words in the substantive portions of the interview as did children interviewed without dolls, and interviewers in the two groups used similar probes to elicit information. The average responses by the children were significantly longer and more detailed when dolls were not used, however. Moreover, children gave longer and more detailed responses to open-ended invitations when dolls were not used.

In an experimental study designed to evaluate the relative effectiveness of two techniques for motivating young witnesses (Mean age = 8.9 years; range, 4 to 12 years) to provide detailed accounts of alleged experiences of sexual abuse, investigators used either of two interview protocols. In half of the interviews, 14 investigators who were naive with respect to the experimental hypotheses used a script containing many open-ended utterances to establish rapport, whereas in the other interviews the same investigators used a script involving many direct questions. Both introductory scripts took about 7 minutes to complete and both included the identical open-ended statement to initiate the substantive phase of the interview. Children who had been trained in the open-ended condition provided 2 1/2 times as many details and words in response to the first substantive utterance as did children in the direct introduction condition. Children in the open-ended condition continued to respond more informatively to open-ended utterances in the later (unscripted) portion of the interview. Seventy-five percent of the children mentioned the core details of the incident in their responses to the first substantive utterance and a further 20% mentioned core details more vaguely.

Significance for Biomedical Research and the Program of the Institute:

Domestic violence is believed to have deleterious effects on children's development. Our research has been unique in its methodological rigor and in the scope of information collected. Ours was the first study in which four different informants--mothers, fathers, teachers, and children--reported on the incidence of the same set of behavior problems using the same measures. The major discrepancies found between reporters underscore the importance in both clinical and research contexts of obtaining information from multiple sources concerning children's adjustment. The findings also suggest that although abused children describe abusive parents more negatively than nonabusive parents, they also describe positive aspects of their relationships with their abusive parents. This information needs to be considered in clinical contexts, such as when child placement decisions are made.

Research on children's testimony is increasingly necessitated by the rapidly increasing numbers of allegations and the lack of reliable and accepted techniques for evaluating allegations, especially those made by very young children. Research focusing on the impact of interviewer style on the quality and quantity of information provided by children is urgently needed in the field, because such research permits the development of improved interview techniques. In addition, research on this topic promises to advance our understanding of children's memory for everyday events, supplementing a literature based on memory for contrived events. Likewise, systematic evaluation of techniques that might help identify plausible accounts is of great practical and forensic importance.

Proposed Course of Project:

Domestic Violence:

We have recently finished re-interviewing the children and parents participating in our longitudinal study. These data, along with data collected in 1993 and 1994 from the teachers and mental health professionals, have been entered into our data base. Data analyses will continue in FY97.

Children's Testimony:

After completing our first systematic validation of the CBCA system we will revise our coding system and expand the study in order to examine in more detail how the application of the CBCA system might differ depending on the child's age, the type of allegation, and the length of delay between the abusive incident and the child's statement. The effects of such factors on the quality of investigative interviews will also be explored. We will also continue field experiments designed to determine how children can best be "trained" to provide richer accounts of their experiences, and whether aids such as anatomically detailed dolls improve or impede children's accounts.

Publications:

Dawud-Noursi S, Sternberg KJ, Lamb ME. Domestic violence in the family context. In: Lewis M, Feiring C eds. Families, risk, and competence. Mahwah, NJ: Erlbaum, in press.

Horowitz SW, Lamb ME, Esplin PW, Boychuk TD, Krispin O, Reiter-Lavery L. Reliability of crieria-based content analysis of child witness statements. Legal and Criminolgical Psychology, in press.

Horowitz SW, Lamb ME, Esplin PW, Boychuk TD, Reiter-Lavery L, Krispin O. Establishing ground truth in studies of child sexual abuse. Expert Evidence, 1995;4:42-51.

Lamb ME. Review of "The book of David: How preserving families can cost children's lives". J Marr Family, in press. [Book Review]

Lamb ME, Hershkowitz I, Sternberg KJ, Esplin PW, Hovav M, Manor T, Yudilevitch L. Effects of investigative utterance types on Israeli children's responses. Internat J Behav Develop, in press.

Lamb ME, Sternberg KJ, Esplin PW. Making children into competent witnesses: Reactions to the amicus brief in re Michaels. Psychol, Public Policy, Law 1995;1:438-449.

Orbach Y, Lamb ME, Sternberg KJ, Williams JMG, Dawud-Noursi S. The effect of domestic violence on children's retrieval of autobiographical memory. In: Williams LM, Banyard VL eds. Trauma and memory. Thousand Oaks, CA: Sage Publications, in press.

Roberts KP, Blades M. Children's discrimination of memories for actual and pretend actions in a hiding task. Brit J Develop Psychol 1995;13:321-334.

Roberts KP, Blades M. Children's eyewitness testimony for real-life and fantasy events. Investigative and Forensic

Decision-Making. Leicester, UK, British Psychological Society, in press.

Roberts KP, Blades M. Do children confuse memories of TV programmes and real life in their testimony? Forensic Update 1995;41:18-22.

Sternberg, KJ. Fathers, the missing parents in research on family violence. In: Lamb ME ed. The role of the father in child development (3rd ed.). New York: Wiley, in press.

Sternberg KJ, Dawud-Noursi SD. Effects of domestic violence on children's behavior problems: Multiple perspectives. In: Tessier R, Tarabulsy GM eds., Child and Family: Contexts for development. Quebec City, Quebec, QU: Les presses de l'Universite Laval, in press.

Sternberg KJ, Lamb ME, Dawud-Noursi S. Understanding domestic violence and its effects: Making sense of divergent reports and perspectives. In: Holden GW, Geffner R, Jouriles EW eds. Children exposed to family violence. Washington, DC: American Psychological Association, in press.

Sternberg KJ, Lamb ME, Dawud-Noursi S. Using multiple informants and cross-cultural research to study the effects of domestic violence on developmental psychopathology: Illustrations from research in Israel. In: Luthar SS, Burack JA, Cicchetti D, Weisz, J eds. Developmental psychopathology: Perspectives on risk and disorder. New York: Cambridge University Press, in press.

Sternberg KJ, Lamb ME, Hershkowitz I. Child victims and witnesses in Israel: Evaluating innovative practices. In: Bottoms BL, Goodman GS eds., International perspectives on child witnesses. Thousand Oaks, CA: Sage Publications, 1996;62-76.

Sternberg KJ, Lamb ME, & Hershkowitz I. Child sexual abuse investigations in Israel. Crim Just Behav, 1996;23:322-337.

Sternberg KJ, Lamb ME, Hershkowitz I, Esplin PW, Redlich A, Sunshine N. The relationship between investigative utterance types and the informativeness of child witnesses. J App Develop Psychol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 01116-09 LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Social Development Across the Lifespan in Diverse Cultures and Ecologies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	M. E. Lamb	Head, SSED	LCE, NICHD
Other:	K. J. Sternberg	Research Psychologist	LCE, NICHD
	C. Bassen	NRSA Fellow	LCE, NICHD
	S. Noursi	IRTA Fellow	LCE, NICHD
	J. Solotaroff	Tech-IRTA Fellow	LCE, NICHD

COOPERATING UNITS *(if any)*

U. of NC, Greensboro (Hattie, MacKinnon-Lewis); U. of MD, Baltimore (Serpell); Ecole Normale, Cameroon (Nsamenang); Hebrew University, Jerusalem (Greenbaum); J. Pearlman; P. Domenici-Lake; L. Baradaran.

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Section on Social and Emotional Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves a number of studies carried out in a variety of different cultures. The overall objective is to explore the ways in which developmental environments can be described by variations in physical and social ecologies, especially in terms of parental beliefs, values, and practices, and to assess how differences in these domains affect children's development. In one study, researchers are attempting to assess specific maternal and child attributions about one another in order to identify the extent to which attributions or expectations shape the way that parents and children interact, with particular focus on the development of aggressive behavior. Mothers and sons interact more aggressively when they have negative attributions of one another; recent analyses suggest that maternal and filial attributions also affect future aggressive behavior. In a second study, Palestinian mothers, grandmothers, and great-grandmothers are being interviewed about their perceptions, values, expectations, responsibilities, and practices. The goal is to explore transgenerational changes in childrearing practices and beliefs in a culture exposed to tremendous exogenous influence, especially over the last half century. In a third study, gender differences in the self-perceptions of young adolescents are being assessed so that staff can explore the antecedents and correlates of different styles of self-perception.

Project Description:

Objectives:

Human development is the result of a complex interplay of multiple influences. Just as there is much variability in human physiognomy across races and ecologies, so too is there remarkable variety in childrearing patterns across cultures and ecologies. An ecocultural research perspective on child care is likely to provide unique insights for understanding how diverse cultures cope with the childcare challenges they face and how they actually design, provide, and ensure the provision of childcare. The short-term objectives of the project are to identify and explore the various properties that characterize different rearing ecologies and to describe some of the intrapsychic processes that may be influenced by environmental variations. The project's long-term goal is to compare and contrast developmental processes across developmental niches in order to identify universal or transcultural principles that underlie the development of human beings. To address these broad goals, studies have been undertaken to describe childrearing contexts, the dynamics of intrapsychic processes, and the processes whereby parental beliefs affect parental behavior and thus children's development.

In one study, initiated in 1990, procedures were developed to assess mothers' and children's attributions about one anothers' intent. Individual differences in these attributions were expected to predict differences in the coerciveness of their interactions. Cross-sectional and longitudinal studies were designed to explore these associations developmentally.

The most recently initiated studies are concerned with gender differences. In one, the female members of three-generational Moslem families in Palestine are being interviewed to explore changing conceptions of gender differences and their implications for socialization in a rapidly changing sociocultural context. The Moslem-Palestinian population in Israel provides a unique opportunity for studying parental ideas and socialization practices within a society in transition. Traditional Arab society prescribes explicit gender differentiation, with boys raised to become independent wage earner while girls are prepared to become mothers and housewives. The Palestinian population in Israel is undergoing continuing changes in family life-style because of urbanization, industrialization, and socio-political conflicts which challenge the traditional child-rearing practices. The rapidly unfolding transition from a traditional to a modern life-style makes it possible to study the changes occurring in beliefs and child-rearing practices across time. We predicted that maternal ideas and socialization practices would vary across generations, that there would be a clear distinction between maternal ideas and socialization practices in relation to boys and girls, and that these gender differences would be greater among mothers in the older generations.

For another ongoing study, procedures were developed to study gender-linked aspects of self-identity, as well as the family and cultural antecedents of difficulties with self-identity among young adolescents. The research is designed to address Gilligan's theory that there are profound differences in male and female senses of self and maturity, as well as gender differences in the "fear of success".
Cross-cultural studies further suggest that certain stereotypes about gender differences are nearly universal: adaptive instrumental traits relating to activity and assertion are associated with men, while integrative-expressive traits having to do with emotions and relating to others are associated with women. However, studies of psychological gender differences have yielded mixed and inconclusive results. It seems likely that self-concept varies according to role; therefore recent studies have examined the effects of gender on self-representation in different roles. Adolescence is a period of individuation, in which one's developing identity is affected by pubertal changes which promote an acute awareness of gender. We studied 7th and 10th graders to assess the prediction that there are gender differences in adolescents' self concepts, which increase from early to mid-adolescence and vary according to role.

Methods Employed:

This project comprises several studies in diverse settings that possess distinctive ecocultural features. During FY96, effort was expended on three studies: One concerned with the effects of maternal and filial beliefs about one another on the quality of their interactions, and the others with gender-related differences in parental beliefs, socialization practices, and self-perceptions.

In the first study, collaborators in North Carolina questioned mothers and their 7-9 year old sons concerning their expectations, beliefs, and attributions about one another and then observed the mother-child dyads in semistructured interactions designed to elicit cooperative or competitive strategies. These procedures were repeated a year later. In addition, teachers described the children's classroom performance and their interaction with peers. Using both cross-sectional and longitudinal data analyses, the goal is to examine the extent to which negative attributions and expectations of hostile behavior contribute to the development of aggressive behavior on the part of young children from diverse socioeconomic backgrounds. Data reduction is now complete and analyses are in progress. The initial (cross-sectional) analyses involved 104 mothers and sons; in the second (longitudinal) set of analyses, some 240 mothers and sons were each interviewed and observed twice, one year apart.

The second study involved open-ended and structured interviews with 60 great-grandmothers, grandmothers, and mothers in three-generational Moslem families in Abu-Ghosh, an Arab village near Jerusalem. The interview format was finalized after extensive pilot-testing in other Arab villages. Staff interviewed informants using a protocol developed for this study; interviews were tape recorded for later coding and analysis. We explored gender differences in maternal beliefs and reported child-rearing practices and the extent to which these beliefs and socialization practices remained constant or changed across three generations of Palestinian women. We predicted that maternal ideas and socialization practices would vary across generations, that there would be a clear distinction between maternal ideas and socialization practices in relation to boys and girls, and that these gender differences wuld be greater among mothers in the older generations.

In the third study, 109 12- to 15-year-old adolescents (7th and 10th graders) of both genders (a) responded to two story stems concerning conflicted accounts of success, (b) completed a questionnaire concerned with guilt and reparation, and (c) described themselves in a variety of roles and contexts ("as I usually am", "as I would like to be", "as I am with my mother", etc.), using 50 self-descriptive adjectives chosen from a list presented to them. Two-thirds of the subjects were or will soon be reassessed one year later to permit examination of developmental stability and change.

Major findings:

In the first study, both maternal and child attributions, as revealed in interviews about one another, were significantly related to the coerciveness of their interactions, as evident in two game-like tasks (Trouble, Etch-A-Sketch). The most aggressive dyads were those in which both mothers and sons perceived hostile intent in the other. Mothers' (but not sons') attributions were also related to teachers' reports of the children's aggression in the classroom. Boys who reported many stressful events in their lives behaved coercively with their mothers and were viewed by their teachers as more aggressive and less socially competent with peers. The relation between stressful life events and the boys' reported aggression with their peers was mediated, in part, by the boys' coerciveness with their mothers. The association between boys' coerciveness with their mothers and social acceptance by peers appeared to be mediated by the aggressiveness of the reported interactions with peers.

Longitudinal analyses suggested that boys' earlier aggression was not predictive of more negative subsequent attributions in mothers, whereas mothers' negative behavior indeed predicted subsequent negative biases on the part of the boys. However, even after considering both mothers' and children's earlier negative behavior, children's negative attributions about their mothers contributed significantly to the aggressiveness of their subsequent behavior with their mothers. The same was not true for mothers: mothers' earlier attributions only indirectly influenced their subsequent aggressive behavior with their sons.

Three generations of Palestinian mothers had surprisingly similar views of gender differentiated practices and behavior, with mothers in all three cohorts maintaining traditional views and practices. As predicted, maternal ideas and socialization practices were found to be different for boys and girls on several variables: age of playmates, types of daily activities, location of daily activities, maternal satisfaction with children's caretaking, maternal dissatisfaction with children's lying and wandering, importance attached to marriage, ideal age of marriage, and ideal number of boys versus girls in the family. Contrary to prediction, however, differences were found on only a few variables among the ideas and socialization practices of mothers from the three generations: children's engagement in watching television, location of daily activities, maternal reactions to girls' caretaking of their siblings, and maternal perceptions of the ideal age for marriage. Thus, contrary to expectations, it did not appear that contact with the modern Israeli society had affected the basic values and beliefs of women in this one Arab village. Qualitative coding and data analysis are now in progress.

In the research on adolescents, internally reliable multi-item measures of affiliation, assertion, and negative affiliation (traits that interfere with closeness) were constructed to assess whether self-ratings varied according to gender, age, and/or role. As predicted, girls reported being significantly more affiliative than boys did, where boys rated themselves higher on the negative affiliative scale. There was no evidence that gender differences increased from 7th to 10th grade. Contrary to prediction, there was little evidence of gender differences for assertion when combined role ratings were employed. However, the effect of gender was significant when assertion was analyzed according to role. Although there was no gender differences for 10 of 11 roles, boys' self-ratings on the assertive scale were higher than girls' for "myself as a boy/girl." Self-ratings varied significantly according to role on all 3 scales, and sex-role interactions were also significant. Boys' and girls' self-ratings tended to change in opposite directions when they pictured themselves in gendered roles: "Myself as a Boy" was the most assertive and the most negative affiliative role; in contrast, girls' ratings on these scales were higher for "Myself as I Usually Am."

Significance for Biomedical Research and the Program of the Institute:

The significance of the project lies in its focus on disparate cultures in an attempt to elucidate normative developmental processes. By obtaining data about development in diverse Western and non-Western societies, the results extend our knowledge of development and underscore the need to understand the broader socio-cultural contexts in which development takes place. This will enable us to characterize the relationship between environments and developmental processes, which is especially important at this time of increasing complaints about the monocultural nature of developmental psychology, as well as concern about the limited universality of developmental norms and principles. Similar concerns apply to the historical preoccupation with the development of males.

Future Goals:

Longitudinal data gathered on parental and child beliefs, attributions, and behavior in Greensboro, NC will be used to explore patterns of mutual influence and change over time, as well as the relationships between attributions and later peer acceptance. In addition, plans are being made to follow these children, and a comparable group of girls, as they move into adolescence. Analyses of gender-related differences in self-identity will be completed in FY 97, as will the preliminary review of the Arab women's attitudes and beliefs. Gender-related differences in self-identity will next be studied in other sociocultural contexts.

Publications:

Hwang CP, Lamb ME, Sigel I. eds, Images of childhood. Mahwah, NJ: Lawrence Erlbaum Associates, 1996.

Lamb ME. L'influence du pere sur le developpement de l'enfant. [Paternal influences on child development]. Enfance, in press [French].

Lamb ME. ed, The role of the father in child development (Third edition). New York: Wiley, in press.

Lamb ME. The role of the father in child development: An introduction and overview. In: Lamb ME ed, The role of the father in child development (3 e). New York: Wiley, in press.

Lamb ME. The development of father-infant relationships. In: Lamb ME ed, The role of the father in child development (3 e). New York: Wiley, in press.

Lamb ME. Review of "Family, justice, and delinquency." Fam Rel, in press.

Lamb ME. Fathering in America: New challenges and champions. Contemp Psychol, in press.

Lamb ME. Review of "Divergent realities: The emotional lives of mothers, fathers and adolescents." Soc Service Rev 1996;70:489-490

Lamb ME. Review of "Fatherless America: Confronting our most urgent social problem." J Marr Fam 1996;58:526-527.

Lamb ME. Paternal influences on child development. In: van Dongen MCP, Frinking GAB, Jacobs MJG eds, Changing fatherhood: An interdisciplinary perspective. Amsterdam, The Netherlands: Thesis Publishers, 1995;145-157.

Lamb ME, Billings LL. Fathers of children with special needs. In Lamb ME ed, The role of the father in child development (3 e). New York: Wiley, in press.

Lamb ME, Hwang CP. Images of childhood: An introduction. In: Hwang CP, Lamb ME, Sigel I eds, Images of childhood. Mahwah, NJ: Lawrence Erlbaum Associates, 1996;1-12.

Nsamenang, AB & Lamb ME. The force of beliefs: How the parental values of the Nso of Northwest Cameroon shape children's progress toward adult models. J Appl Develop Psychol 1995;16:629-643.

Volling BL, MacKinnon-Lewis C, Rabiner D, Baradaran LP. Children's social competence and sociometric status: Further exploration of aggression, social withdrawal, and peer rejection. Develop Psychopath, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01117-09 LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Behavioral Pediatrics: Coping with Medical Experiences and Development under Cocaine Exposure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M. H. Bornstein	Head	LCE, NICHD
J. L. Genevro	IRTA Fellow	LCE, NICHD

COOPERATING UNITS *(if any)*

Z. N. Kain, Yale University
L. C. Mayes, Yale University

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Section on Child and Family Research

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research in behavioral pediatrics examines, first, contributions of developmental and individual-differences factors to children's understanding of routine medical care and of strategies for coping with distressing medical experiences and, second, child development under cocaine exposure. The research on children's understanding of and responses to medical care integrates the adult stress and coping literature, research on changes in children's cognitive capabilities as they mature, literature pertaining to preparing children for hospitalization, and recent theoretical work regarding the effects of routine pediatric health problems and treatment on child development. The research on development under cocaine exposure addresses issues central to understanding ongoing transactions between biological factors and environmental conditions as they relate to various developmental outcomes. A third focus of collaborative behavioral pediatrics research is on factors related to the incidence of injuries in young children in full-time child care.

Project Description:

I. Medical Experiences and Hospitalization in Children

The primary goal of this research is to determine how developmental processes (e.g., changes in cognitive and emotion-management capabilities) and individual difference factors (e.g., gender and problem-solving ability) influence children's understanding, emotions, and behaviors in routine and aversive medical care situations. In this research, social-cognitive developmental theory is applied to the investigation of children's understanding of medical care and strategies for coping with distressing medical experiences, and children's capabilities in the face of stresses related to their physical well-being. Specific research questions include how cognitive developmental levels influence (1) children's comprehension of routine pediatric health care experiences and (2) their knowledge of, preferences for, and use of strategies for coping in stressful medical situations. The project also examines complex relations between coping and variables such as children's problem-solving ability, gender, anxiety, and parental anxiety that may moderate or mediate relations between children's age and coping knowledge and behavior.

Three major studies have been conducted, the first on children's opinions; the second on children's reactions before hospitalization, on the threshold of surgery, and after hospitalization; and the third on cognitive and affective dimensions of young children's mental representations of routine medical care. Two additional studies are in process: The first, conducted with collaborators at the Yale University School of Medicine, is in the data collection phase; the second is a study of health and development in participants in the longitudinal study of maternal-child interaction ongoing in the CFRS.

The first study, on children's opinions, involved school-aged children not experiencing or facing any unusually stressful events. In this study, for which data collection and analyses have been completed, 75 children between 5 and 12 years who were not anticipating hospitalization or surgery were interviewed concerning their knowledge of coping. Coding schemes were developed for categorizing the coping strategies proposed by subjects. Children's responses were grouped into categories based on coping techniques discussed in the adult coping literature, on a continuum from approach to avoidance techniques, with emotion-manipulation techniques lying equally between these two poles. Avoidance tactics, the main focus of interest for the management of uncontrollable stress (such as hospitalization), were further divided into partial (cognitive and behavioral distraction) and complete (escape and denial) types. Cognitive distraction includes fantasy and thinking about something else; behavioral distraction includes playing and watching television; escape is leaving the situation or going to sleep; and denial involves suppressing and forgetting. These coding schemes were later used to analyze children's responses to the coping interviews in the hospitalization study. Identical coding schemes were used for both investigations to facilitate the comparison between children's coping assessed in neutral settings and coping assessed in a real-life stressful situation.

Participants in the second study, which focused on children's reactions to hospitalization, were 62 children between the ages of 5 and 13 years hospitalized for elective surgery in a large, inner-city hospital and their parents. This study is unique in that children and parents were observed and interviewed at multiple time periods before, during, and after hospitalization: (1) one week before a pre-admission program, (2) the morning of the pre-admission program, (3) the day of admission, (4) the morning of surgery (before leaving the ward to go to the operating room), (5) waiting outside the operating room, (6) entering the operating room, (7) the morning after surgery, (8) one-week after surgery, and (9) one month after surgery. Self-report, parent-report, and observational measures were used to assess aspects of children's knowledge of coping strategies, information seeking, expectations, level of anxiety, and behavioral responses to the experience.

The third study, which investigated the nature of children's representations of routine medical care, included 70 children and their mothers. Children were interviewed at 4 year of age. Transcripts of children's knowledge of routine pediatric care events were coded for cognitive structure and content. Transcripts were also coded for emotion content. Measures completed by each mother included assessments of the child's health status and the mother's preferred methods of treating her child's physical symptoms. The latter included ratings of the degree of concern/worry each mother felt about her child's health, relative to her perceptions of other mothers of children the same age, and her perceptions of her child's general health.

The first three studies are complete. Coding schemes for several open-ended measures in these studies have been developed, and protocols for those measures have been coded.

Although information about children's knowledge and use of distraction strategies is slowly accumulating, little is known about children's perceptions of the usefulness of distraction strategies in helping them cope with different types of distress. Our research on children's coping has focused on children's knowledge of strategies for coping with potentially distressing experiences in which avoidance coping strategies may be particularly useful. As a result of this research, we have learned about the coping strategies school-aged children are able to articulate in response to questions about coping in hypothetically stressful situations. Less is known, however, about children's estimations of the value (how good a strategy is for dealing with a distressing situation) and efficacy (whether the strategy will change feelings of fear and/or distress) of specific strategies.

We have therefore investigated children's perceptions of the value and efficacy of strategies for coping with uncontrollable stressful situations, given findings in the adult literature that certain types of avoidance strategies have been characterized as more or less effective. Because conclusions about which strategies work best for adults have often provided the foundation for programs designed to help children cope with stressful medical experiences, it is important to determine the ways in which children's perceptions differ from or model findings from research with adults. In addition, the possibility that the experiences of being hospitalized and having surgery affect children's perceptions of the value and efficacy of specific strategies was investigated. Our work in this area was guided by these questions: (1) How valuable do children consider different strategies for coping with the stresses associated with undergoing medical treatment? (2) Which strategies do children think are likely to change feelings of fear about undergoing medical treatment? (3) Do children's perceptions of the value and efficacy of coping strategies differ as a function of age? (4) Does age predict children's reports of strategy use? (5) Do children's estimations of strategy value and efficacy predict which strategies they report having used while hospitalized? (6) Do children's perceptions of the value and/or efficacy of specific strategies change as the result of being hospitalized? (7) Do children's perceptions of strategy value and efficacy change based on strategy use while they were hospitalized? In addition to proposing these general questions, we specifically hypothesized, based on related research on children's coping and problem-solving abilities (e.g., Altshuler, Genevro, Ruble, & Bornstein, 1995), that age would relate positively to children's perceptions of the value and efficacy of cognitively mediated avoidance strategies (e.g., cognitive distraction). Because being hospitalized and having surgery are experiences over which children typically can exert little or no control, we also hypothesized that estimations of the value and efficacy of specific emotion-focused strategies (behavioral distraction and cognitive distraction) would increase from pre- to posthospitalization. Finally, based on previous related research on uncontrollable stressful situations, we anticipated that children would most frequently report using behavioral distraction strategies to cope with feelings of fear while hospitalized.

Analyses indicated that compliance and the availability of social support may be slightly more important coping tactics for children than for most adults, but also that both behavioral and cognitive distraction strategies play highly valued roles in children's repertoires for coping with distressing medical experiences. Results indicated that older children were more likely to consider cognitive distraction a useful strategy, and they were consistent with results of analyses examining children's knowledge of coping strategies. In addition, children who believed that behavioral distraction strategies were likely to change their feelings were more likely to report having used those strategies while hospitalized. Data analyses focusing on age-related differences in children's ratings of the value and efficacy of specific coping strategies in relation to coping by a child in a hypothetical situation and their own coping efforts during hospitalization have been completed.

To examine the genesis of children's knowledge and understanding of strategies for coping with distressing medical experiences, we have also begun to examine the role played by children's representations of their medical experiences as a means by which cognitions about and emotional responses to distressing events may be linked. We examined relations among parental health perceptions, anxiety, and children's mental representations of medical care to investigate the premise that children's mental representations provide an avenue by which parental anxiety may influence children's knowledge and enactment of specific coping strategies. We propose that children's mental representations of routine medical care include not only the events experienced but children's affective responses. These elaborated mental representations, therefore, may serve as a foundation for the development of children's abilities to cope adaptively with distressing medical treatment.

Results from the study of 4-year-olds' mental representations indicated that children's representations included differentiated details about routine pediatric care, including specific events experienced and associated affective responses. We found, as hypothesized, that mothers' concerns about their children's health were related to the content and structure of children's representations of the event of going to the doctor. This finding supports further investigation of the premise that children's mental representations serve as a mechanism by which parental attitudes and behaviors, and children's early experiences with medical care, influence the development of coping knowledge and behaviors. Results of the study also suggest that gender differences in children's representations of routine pediatric care, and in affective responses to that care, should be examined in future research.

Studies in Process

A collaborative study with colleagues at the Yale University School of Medicine and is targeted toward the investigation of preoperative anxiety in children and parents. Subjects are children between the ages of 5 and 12 years who are admitted for outpatient surgery. Measures include the coping interview used with children in the hospitalization studies described above, a coping questionnaire for parents based on a modification of the coping interview, measures of child and parent anxiety, observations of child behaviors during anesthesia induction, and measures of recovery from surgery. Analyses will focus on relations among age, coping strategy knowledge, and parental and child anxiety as predictors of children's behaviors and recovery.

Data collection in a study of children's physical health and development is complete. Medical records of approximately 170 4-year-olds have been coded (information derived includes, for example, total number of visits to pediatricians, emergency rooms; well visits; sick visits; diagnoses; referrals for developmental evaluation, surgeries, etc.; number of illness episodes; duration of illness episodes). Questionnaire data were also obtained from mothers regarding their perceptions of their children's susceptibility to illness and general health status, and their own propensity to medicate their children, seek pediatric care for specific illnesses and conditions, and a number of other factors related to health attitudes and behaviors. Analyses of these data will include investigations of a number of current questions in development and health, such as relations between recurrent otitis media and language and behavioral development, and child and maternal predictors of pediatric health care utilization.

Recently concluded analyses focused on 41 firstborn children for whom complete medical records were available for the full 12-month period immediately prior to a 4-year laboratory follow-up visit: Mothers' general perceptions of their children's susceptibility to illness and their own propensity to seek pediatric care were measured. Mothers' specific perceptions of their children's susceptibility to upper respiratory illness and ear problems, and their own propensity to seek pediatric care for these conditions were also assessed. Sociodemographic characteristics (including maternal age and level of education), maternal anxiety about child health, and past pediatric health care utilization as documented in children's own medical records were evaluated as predictors of mothers' general and specific ideas about their children's susceptibility and their own propensity to seek pediatric care. Results indicated that mothers simultaneously possess general and specific ideas about their children's susceptibility to illness and their own propensity to seek pediatric care, and that these general and specific ideas are differentially predicted by maternal anxiety and the number of recent pediatric visits made by children. These results support the continued examination of specific and general predictors of pediatric health care attitudes and utilization.

Child Development and Behavioral Pediatrics, an edited volume resulting from a multidisciplinary NICHD-sponsored conference on current issues in behavioral pediatrics research which we organized in 1995, is now in press (Lawrence Erlbaum Associates, August, 1996).

II. Cocaine Exposure in Children

The problem of prenatal cocaine exposure is one well-suited to analysis from the perspective of behavioral pediatrics. Research on cocaine exposure involves examining transactions between biological or genetic factors and external environmental conditions as related to patterns of disordered behavior; focusing on individual patterns of adaptation and maladaptation; allowing that individuals may shift back and forth between normal and abnormal modes of functioning as a consequence of differing developmental stressors and environmental conditions; utilizing naturally occurring events or "experiments of nature" to understand the expected developmental ontogeny of specific functions; and employing the conceptual frames and methods of multiple disciplines to study any one mode of function, adaptation, or behavior.

For children exposed to cocaine prenatally or postnatally, the outcome is not one of static impairment or dysfunction, but rather reflects a dynamic transaction between biologic conditions (resulting from prenatal cocaine exposure in the intrauterine environment) on fetal brain development and postnatal environments also shaped by ongoing parental substance abuse. Prenatal cocaine exposure does not appear to exert a singular effect on any one developmental function, nor does any single effect appear to express itself in all infants. Some prenatally cocaine exposed infants and young children develop adaptively and along essentially normal trajectories in their first years of life, a finding that raises important questions about what constitute detrimental prenatal levels of exposure and what the postnatal environmental conditions that mediate positive versus negative effects of prenatal cocaine exposure may be. For the child and parent, prenatal cocaine exposure presents risks for multiple other problems, such as involvement in violence and crime, homelessness, poor school performance as well as early school drop-out, and multi-generational drug abuse, problems that trap both parent and child in poverty and discord. These factors influence children's level of adaptation at any given point in time and may contribute to shifts between adaptive and maladaptive patterns of response over the course of development.

Prenatal cocaine exposure also offers an "experiment of nature" in that the developing fetal brain is exposed to an agent that may have potentially teratogenic effects on specific developmental capacities. In the case of prenatal cocaine exposure, as well as adult substance abuse, capacities that are potentially affected involve the regulation of states of attention and arousal, which in turn may affect information processing, learning, and social relatedness and are sensitive to environmental disruption and discord. Finally, studies of cocaine exposure require a multidisciplinary approach that draws at least on the following fields: neurobiological models of brain development, neuropharmacological data on the effects of cocaine at the level of the neurotransmitter, behavioral teratology, pediatrics, developmental psychology, psychiatry and the treatment of substance abuse, child psychiatry, sociology, and social policy. Understanding the methods for studying prenatal cocaine exposure and the implications of findings in this field calls for the integration of data from each of these disciplines, from animal as well as human models, and from individual or small group studies as well as population surveys. Our studies of prenatal cocaine exposure address these several issues.

One chapter resulting from our work investigating the consequences of cocaine exposure reviewed studies of attentional regulatory mechanisms and relations among visual attention, information processing, and social interaction in groups of infants at risk for central nervous system impairments--infants born prematurely and infants exposed to cocaine prenatally. The capacity to attend to the outside world is fundamental to infants' learning about the world, themselves, and those adults who care for them. When actively attending, infants are processing information, inviting social interactions, and observing and experiencing the effects of their own actions on the inanimate and animate environment. Prerequisite to every theory of learning, and implicit in every theory of social development, are states of arousal (or level of alertness) and attention. For infants and young children, concepts of arousal and attention regulation are closely related. An alert, optimally aroused state is necessary for infants' sustained attention to objects or people, and the capacity to attend in infancy has predictive validity for broader cognitive and social functions. Attention measured in infancy has been shown to relate to exploration, problem solving, and intelligence; and the infants' capacity for sustaining an alert, attentional state supports ongoing and extended interactions with adults.

Investigation of possible differences in attentional regulation in preterm infants and infants exposed prenatally to cocaine are of particular interest because, for both, specific perinatal events make it plausible to hypothesize impairments in attentional regulation. For preterm infants, these perinatal events include the effects of respiratory distress, overall cardiovascular compromise, and complications such as intraventricular hemorrhage on those central nervous system functions serving arousal and attention regulation. In the case of prenatal cocaine exposure, the specific effects of cocaine on monoaminergic neurotransmitter systems and cocaine-related reduction in placental blood flow both may contribute to impairments in arousal and attention regulation. We discussed the concept of attention in infancy. For preterm and cocaine-exposed infants, respectively, we then reviewed demographics, developmental biology, and arousal regulation and attention. Last, we considered remediation in these two at-risk conditions through environmental intervention including changes in parenting behaviors.

Infants normally and naturally show interest in people and objects that are part of their everyday interactions in the world; indeed, infants seem "programmed" actively to seek out stimulation. Once information is familiar, infants just as typically lose some of their interest. Formally, they show a decrement in attending to stimuli that repeat or are continuously available to them. This peaked and waning pattern of attention during interactions of infants with faces and with objects they naturally encounter at home is observed widely. Interest and growing disinterest alike are commonplace reactions to materials that infants came into contact with in the course of daily life. The decrement in attending infants show in such situations is called "habituation". Habituation represents an elementary kind of learning and is a characteristic of babies, and they show considerable individual variation and short-term stability of habituation. Habituation has been interpreted as processing information, and habituation in infants has been shown to play a role in the expression of later childhood cognitive performance. One chapter resulting from our interest in habituation in cocaine-exposed infants reviewed procedural and psychometric issues involved in the measurement of habituation, examined arguments for the information-processing interpretation, surveyed data for the predictive validity of habituation in normal infants, and discussed the origins of habituation as well as the role of experience in its expression. Finally, habituation in at-risk cocaine-exposed newborns and young infants was described.

Our empirical work in this area has included one experimental study designed to investigate the effects of prenatal cocaine exposure on 3-month infant information-processing and developmental assessments. One hundred and eight infants--61 cocaine exposed and 47 controls--participated at 3 months of age in an infant-control habituation and novelty responsiveness procedure and in a developmental assessment using the Bayley Scales of Infant Development both administered by experimenters blind to the drug exposure status of the infant. Compared to the non-drug exposed group, infants exposed prenatally to cocaine were significantly more likely to fail to start the habituation procedure and, for those who did, significantly more likely to react with irritability early in the procedure. The majority of infants in both groups reached the habituation criterion, and among those who did there were no significant differences between cocaine and non-cocaine-exposed infants in habituation or in recovery to a novel stimulus. Infants who were cocaine-exposed showed comparatively depressed performance on the motor (PDI) but not the mental (MDI) scales of the Bayley. These results obtained for habituation and Bayley MDI controlling for both perinatal and sociodemographic factors. It appears that differences in reactivity to novelty, but not in information-processing, between cocaine-exposed and non-cocaine-exposed infants suggest that the effects of prenatal cocaine exposure may fall on arousal and attention regulation rather than early cognitive processes.

We pursued this lead in a separate study that investigated relations between drug exposure, particularly cocaine, and infants' regulation of arousal in response to novelty. Sixty-three infants--36 cocaine-exposed and 27 non-cocaine-exposed--participated at 3 months of age in a novel-repeat stimulus presentation procedure. Arousal was operationalized in terms of infant behavioral state, affective expressiveness, and attention to the stimulus. Infants were tested, and infant behaviors were scored by experimenters blind to the drug exposure status of the infant. There were no differences between the two groups in baseline behavioral state or affective expression before the presentation of novel stimuli. Compared to the non-cocaine exposed group, infants exposed prenatally to cocaine and other drugs were more likely to exhibit a crying state and to display negative affect on novel stimulus presentations. There were no group differences in the amount of looking toward the stimulus. Both groups showed less crying and negative affect when stimuli were presented a second time, but decrements were consistently greater for the cocaine-exposed group. These results obtained when group differences were controlled for sociodemographic and perinatal variables. Sources of differences in the regulation of arousal in cocaine-exposed and non-cocaine-exposed infants were discussed, and impairments in the regulation of arousal in cocaine-exposed infants were considered in a framework of predictive implications for children's social and cognitive development.

Prolonged cocaine addiction by its very intractable nature defines a way of life, and popular notions about the lifestyle of cocaine abuse have always abounded in stereotypes. One view persisting from the late 19th century portrays the innocent or eccentric but wildly imaginative individual jump-starting his creative slumps with an occasional, absolutely controlled, if not misguided, use of cocaine. The other, currently more popular, stereotypic extreme is the sinister, dangerous derelict whose life has been self-destructed by irresponsible cocaine abuse. These are the individuals whom communities abhor, enact laws against, and pursue. Similar negative stereotypes about cocaine use are seen in images of parents who abuse cocaine and in portrayals of children who were exposed to cocaine prenatally. That is, no one abusing cocaine can care for children, and children exposed to cocaine prenatally are universally and irrevocably damaged. However, how cocaine use actually affects mothers' attentiveness and responsiveness to their infants during interactions has not been extensively studied. We compared the face-to-face interactions of 43 cocaine-using mothers and their infants at 3 and 6 months to 17 non-cocaine but other-drug-using mothers and 21 mothers who used no drugs during their pregnancy. Coders blind to the mothers' drug use status scored 3 min of face-to-face interactions for 16 measures of maternal and infant interactive behaviors. A principal component of 7 behaviors formed a measure of maternal attentiveness; a principal component of 5 behaviors formed a measure of mother-infant dyadic organization; and a principal component of 4 behaviors formed a measure of infant readiness to interact. A measure of maternal interruption was computed as the mean standard score of 3 additional interruptive behaviors. At 3 and 6 months, cocaine-using mothers were less attentive to interactions, and cocaine-using mothers and their infants engaged in fewer dyadic interactions than either non-cocaine or non-drug-using mothers. Compared to 3 months, cocaine-using mothers at 6 months were less attentive to interactions and more frequently interrupted interactions by looking away, redirecting the infant, or withdrawing, whereas non-cocaine-using and non-drug-using mothers showed no change or an improvement in attentiveness to interactions and a decrease in interruptions. No differences emerged in the interactive behaviors of the infants of cocaine-using, non-cocaine-using, or non-drug-using mothers, but by 6 months, the infants of cocaine-using mothers were less interactive than either the infants of non-cocaine-using or non-drug-using mothers. Cocaine use appears to represent a significant risk for diminished parental attentiveness and responsiveness to infants.

Current work is focused on examining the potential longer-term effects of prenatal cocaine exposure and continued cocaine used by mothers on developmentally salient tasks and behaviors in the toddler and preschool periods. Preliminary analyses of 18-month-old children's play activities with their mothers indicate, for example, no significant differences in the frequency or duration of symbolic play by mothers or children among groups of cocaine-exposed, other-drug exposed, and non-drug-exposed children. Analyses are proceeding to determine whether differences exist between these groups in patterns of mother-child dyadic interaction during play, as reflected in the emotional availability of mothers and children, or in children's language acquisition and use.

III. Other Collaborative Projects:

Other behavioral pediatrics research projects include ongoing collaborations with investigators in the School of Public Health at the University of California, Berkeley, and the School of Medicine at the

University of California, San Francisco. At present, this work focuses on the investigation of environmental and individual factors predicting injuries in young children in full-time child care. Recent analyses indicate that environmental factors such as the occurrence of stressful events in childcare centers, and individual differences factors such as psychobiologic reactivity and emotional and behavioral problems, interact to predispose some children to injury even in high-quality child care environments.

Significance of Biomedical Research and the Program of the Institute:

Developmental and psychological factors are central elements in many problems in child health, as for example, in children's understanding of and coping with medical procedures and children's exposure to cocaine. Current theories of child development as a continuing transaction between children's inherent characteristics and the multiple environments in which they develop provide a foundation for identifying and understanding the ways in which children's physical health and development are intertwined. The transactional view holds that the effects of an experience depend on the nature of the specific experience and the constitutional endowment of the child. Similarly, the individual's contribution to his or her own development at any point reflects endogenous characteristics in combination with aspects of that individual's life history. This perspective emphasizes the ways in which biologically-based propensities and individual experiences mutually influence development. Neither biological predispositions nor experiences alone determine the course, direction, termination, or final resting level of development; rather, these life forces influence one another as development proceeds. The transactional model of development thus can be applied to the analysis and understanding of medical problems in children. From child development comes the view that maturational status, developmental level, experiential history, and individual differences factors (like intelligence, gender, temperament, and so forth) coordinate to engender differential reactions and responses to everyday and severe medical challenges. From behavioral pediatrics comes the view that biological, physical, and epidemiological factors converge to challenge the child medically. It is the task of child development and behavioral pediatrics to coordinate these joint perspectives on children's health problems, and it is toward this end that the behavioral pediatrics research program of the CFRS is oriented.

I. Medical Experiences and Hospitalization in Children

As previous findings indicate that young children are often unable to manage pain or fear on their own, it is important to determine whether parents and hospital staff can effectively bolster children's efforts to manage their emotions in distressing and painful medical treatment situations. In order to ascertain which coping tactics are favored by children in specific situations, which tactics are most effectively used by which children under what circumstances, and who children consider to be effective "coping partners," new interview measures of children's coping knowledge and expectations are being developed based on the findings of these studies. The identification of the effects of variables, such as problem solving, gender, and anxiety, on coping and on relations between age and coping, also may make it possible to more successfully individualize programs preparing children for stressful medical procedures. The findings, both extant and emerging, of this project hold the promise of contributing in important ways to refining theories of cognitive development and coping. The effects of development on coping -- and the influences of coping on development -- are important pieces of the puzzle of relations between challenge and adaptation. The convergence in this project of cognitive-developmental theory with issues in the practice of behavioral pediatrics expands the realm of possible contributions of this research program. These potential contributions include measures to be used in other NICHD research, enhancements of the treatment of hospitalized children based on developmental theory, and revision of developmental theory based on the study of children in not uncommon stressful situations.

II. Cocaine Exposure in Children

A focus on young children from cocaine-abusing families represents a significant area of study for behavioral pediatrics. Some major cities in the United States report 50% of women receiving prenatal care at inner city hospital clinics have used cocaine regularly during their pregnancy and for several years before. Further, an increasing number of children are born to cocaine-using women and/or grow up with substance abusing parents: No one estimate is certain, but currently perhaps more than 350,000 infants are born each year in the United States to mothers who have used cocaine with or without other substances (such as alcohol, marijuana, or tobacco) throughout their pregnancy. These twin trends have, in turn, led to two expanding areas of research, the first being a focus on the effects of prenatal cocaine exposure on infants' and children's development, and the second on studies of the characteristics of women who abuse substances and the effects of that substance abuse on their ability to parent. Children from cocaine-abusing families have multiple unmet developmental and health care needs, and their parents often have great difficulty meeting those needs or accessing care for themselves. When we look beyond the stereotypes and popular opinions, the problem of childhood cocaine exposure is marked by multiple levels of interaction between pre- and postnatal factors and between biologic vulnerabilities and disordered environments. Investigators seeking to understand further the effects of cocaine exposure on children's psychological development face the dilemma that cocaine affects development through multiple pathways. Our studies examine the pathways through which prenatal cocaine exposure and ongoing parental cocaine use can affect both child and family. Understanding the developmental and physical needs of children from substance abusing families along with the needs of their parents will inform the development of more integrated health care services for children from substance abusing families.

Proposed Course of Project:

I. Medical Experiences and Hospitalization in Children

Data collection has been completed for three studies of children’s coping and understanding of medical experiences. Data for the first phase of studies of children’s physical health and development have also been collected. Data collection in the collaborative study of children’s coping with elective at the Yale University Medical School is underway. Results of analyses conducted to date suggest that relations between age and coping are affected in complex ways by individual differences factors such as gender and level of cognitive development. Based on these results, new interview measures of children's coping knowledge and expectations have been developed. Analyses of children’s representations of routine medical care suggest that both cognitive and affective factors play central roles in children’s perceptions of their medical experiences. Additional analyses suggest that mothers hold health attitudes and beliefs that are both specific and general, and that specific and general attitudes are differentially predicted by previous pediatric health care utilization. Analyses, both continuing and planned, are designed to: (1) further investigate the role played by children's mental representations of medical care in the development of coping knowledge, coping behaviors, and adaption to distressing medical experiences; (2) investigate relations between physical health status and developmental processes and outcomes; (3)

pursue the investigation of relations among parents' health care attitudes and beliefs, children's health status, and the utilization of pediatric health care; and (4) examine in a more differentiated way than has previously been possible relations among parental anxiety and conceptions of coping, and children's coping knowledge, behaviors, and anxiety prior to outpatient surgery.

II. Cocaine Exposure in Children

Data collection for the longitudinal aspects of this research program continues, and will make it possible to examine the potential effects of prenatal cocaine exposure on a number of different developmentally important outcomes. Ongoing data collection will also investigate the potential effects on children of prenatal cocaine exposure in combination with specific types of chronic environmental deprivation and/or continuing drug use by caregivers.

Publications:

Altshuler JL, Genevro JL, Ruble DN, Bornstein MH. Children's knowledge and use of coping strategies during hospitalization for elective surgery. J Appl Develop Psych 1995;16:53-76.

Bornstein, MH, Genevro JL. eds. Child Development and Behavioral Pediatrics.
Mahwah, NJ: Lawrence Erlbaum Associates, 1996.

Bornstein MH, Genevro JL. Child development and behavioral pediatrics: An introduction. In: Bornstein BH, Genevro JL, eds. Child Development and Behavioral Pediatrics. Mahwah, NJ: Lawrence Erlbaum Associates, 1996;1-9.

Bornstein MH, Mayes LC., Tamis-LeMonda CS. Habituation, information processing, mental development, and the threat of cocaine exposure in infancy. In: Hepper PG, Kendal-Reed M, eds. Perinatal Sensory Development: Psychology and Psychobiology. Cambridge, England: Cambridge University Press, 1996;xx-xx.

Genevro JL, Andreassen CJ, Bornstein MH. Young children's understanding of routine medical care and strategies for coping with stressful medical experiences. In: Bornstein MH, Genevro JL, eds. Child Development and Behavioral Pediatrics. Mahwah, NJ: Lawrence Erlbaum Associates, 1996;59-83.

Mayes LC, Bornstein MH. Developmental dilemmas for cocaine-abusing parents and their children. In: Lewis M, Bendersky M. eds. Mothers, Babies, and Cocaine: The Role of Toxins in Development. Hillsdale, NJ: Lawrence Erlbaum Associates, Publishers, 1995;251-72.

Mayes LC, Bornstein MH. Attention regulation in infants born at risk: Prematurity and prenatal cocaine exposure. In: Burack J, Enns JT, eds. Attention, Development, and Psychopathology. New York: Guilford Press, 1996;xx-xx

Mayes LC, Bornstein MH. The context of development for young children from cocaine-abusing families. In: Kato P, Mann T. eds. Health Psychology of Special Populations. New York: Plenum Press, 1996;xx-xx.

Mayes LM, Bornstein MH. The development of children exposed prenatally to cocaine. In: Luthar S, Burak J, Cicchetti D, Weisz J. eds. Developmental Psychopathology: Perspectives on Risk and Disorder. New York: Cambridge University Press, 1996;xx-xx.

Mayes LC, Bornstein MH, Chawarska K, Granger RH. Information processing and developmental assessments in three-month-old infants exposed prenatally to cocaine. Pediatrics 1995;*95*:539-545.

Mayes LC, Bornstein MH, Chawarska K, Haynes OM, Granger RH. Impaired regulation of arousal in 3-month-old infants exposed prenatally to cocaine and other drugs. Develop Psychopath 1996; 8:29-42.

Mayes LC, Feldman R, Haynes OM, Bornstein MH, Granger RH, Schottenfeld, R. Interactions between cocaine-abusing mothers and their 3- and 6-month-old infants. Inf Behav Develop,

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01119-09 LCE

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Specificity of Mother-Infant Interaction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. H. Bornstein	Head, CFRS	LCE, NICHD
J. Suwalsky	Research Psychologist	LCE, NICHD
K. Painter	Research Psychologist	LCE, NICHD
M. Fivel	Research Psychologist	LCE, NICHD
C. Andreassen	IRTA Fellow	LCE, NICHD
C. Rahn	Research Psychologist	LCE, NICHD
M. Haynes	Statistician	LCE, NICHD

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Comparative Ethology

SECTION
Child and Family Research Section

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.6	1.8	2.8

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project investigates endogenous and exogenous factors that contribute to the development of cognitive and social competencies in children during the first four years of life. Before children are old enough to enter formal social learning situations, nearly all of their experiences stem directly from interactions they have with their primary caregivers. The present study focuses on the extent to which several maternal characteristics (age, parenthood status, SES, parity, employment status, as well as type of substitute care experienced during mother's employment) influence observed relations between caregiving on the one hand and child developing cognitive and social competencies on the other. In this longitudinal study, subjects are visited when they are 5, 20, and 48 months of age. At 5 months, mother-infant interaction is observed. At 20 months, measures of toddler functioning (play competence, language development, and social adaptation) and maternal behavior (play and intelligence) are obtained. At 48 months, the follow-up examines diverse children's abilities (representational competence, problem solving, reasoning skills, mathematical ability, language use and discourse, and selected aspects of generally adaptive behavior).

Project Description:

Objectives: This longitudinal project has several aims. The first is to investigate the influences of caregiver behaviors and beliefs on cognitive and social development in infancy and early childhood. Different classes of caregiving are linked to specific concurrent perceptual-cognitive and socio-emotional competencies of children in the first year of life and have certain predictive validity for later periods of childhood. In this investigation samples of mothers and infants varying on characteristics of the caregivers that might differentially influence the behaviors and beliefs of interest are being observed. The present study set addresses questions of whether and to what extent maternal and infant behaviors, and relations between them, are influenced by the following factors: maternal age (adolescent, younger, older), maternal employment status (homemaker, employed), type of substitute care experienced in connection with mothers' employment (one-on-one care in the baby's own home by a single adult, family daycare), parenthood status (mother by birth or by adoption), SES, and birth order of infant (first- vs. secondborn).

A second major aim of the study is to examine aspects of cognitive and social functioning in the child in the second year of life, both in relation to early experiences and as a function of sociodemographic group membership. The literature contains evidence that aspects of maternal behavior in infancy influence the development of cognitive and social skills in young children. Relations between carefully operationalized aspects of maternal behavior in the first and second years and features of the emerging capacities for mental representation in the child (e.g., language acquisition, level of play competence) in the second year support the view that such influences are multidimensional, specific, and complex. In the present sample, infants seen at 5 months are revisited when they are 20 months old, thereby permitting examination of relations between maternal and infant behavior in early infancy and toddler functioning at the end of the second year of life.

The third aim of this longitudinal study involves assessing a broad range of abilities in the child at preschool age. Measures of the following are obtained: the child's I.Q., self-concept, play and exploratory competence, mathematical skills, symbolic representation, language and storytelling skills, drawing and singing abilities, social abilities, and problem-solving skills. Close study of how maternal behavior influences the child's functioning in cognitive and social spheres, both concurrently and longitudinally, promises to contribute importantly to our understanding of more general issues in preschooler development, such as how children make sense of and represent their experiences, how children acquire and use cognitive and social strategies, and how children learn to plan future activities and to use metacognition, self-monitoring, and self-regulation to anticipate task requirements and to evaluate ongoing performance. Although the question of maternal influences on preschooler development is largely unexplored, work with younger children suggests some avenues of approach for accessing these processes.

Ancillary studies. In several supporting and converging investigations in the laboratory, major dependent variables that are examined in the larger longitudinal project are being scrutinized further. In one, language performance and play sophistication have been investigated in a longitudinal study of children seen at 2, 3, and 4 years of age. First, language and play at 2 years of age have been examined under a systematically varied set of experimental conditions: with mother or stranger, in the home or laboratory. This methodological study was designed to help determine the best estimators of individual differences in child language and play abilities. Perhaps, for example, strangers "lower the gain" in children's verbal output. To what degree? How much, relative to a strange situation? In different representational modes (language and play) or just one (language or play)? The results of this study will allow further insight into how social and physical contexts affect the expression of language and play in the young child, and they are intended to inform a reasoned choice of instrument, condition, and metric for future studies designed to assess central issues at the intersection of young children's cognitive and social functioning. In this same study, stability of performance from 24 to 36 months for mother and child with respect to language and play is being assessed.

Further, at 48 months, measures of the child's "theory of mind" have been obtained. Recent research on early development has focused on the young child's understanding that individuals have internal and unobservable desires and beliefs that explain their observable behavior, i.e., a "theory of mind." Antecedents of this knowledge are being evaluated in children's early mental representational ability, as expressed in their symbolic play and language, as well as in parent-child social interaction. Individual differences in the theory of mind of children 4 years of age are also being explored, following the basic hypotheses that children who were more advanced on measures that assess representational abilities early in life (language and play) will be more sophisticated on this task.

Another study, related conceptually to the main longitudinal design, is organized to document the influence of the loss of a major communication channel (deafness) on mother-child interactions and on children's developing language and play performance during the second year of life. This study is designed to explore the role of children's early language learning environments on the development of symbolic activity. Deafness in a family has a profound impact on the child's language-learning environment, and this impact will be mediated by whether the parents or child or both are deaf. For a deaf child, hearing and deaf parents provide significantly different language-learning opportunities and, by the same token, the language-learning environments provided by deaf parents of hearing and deaf children also differ. Language variation among children at the start of the second year has been shown to be associated with variation in their play sophistication, suggesting that these twin emerging abilities might reflect a transition in a common underlying "representational competence." Deafness either of the child or in the family may also be associated with variability in play sophistication. Such variability, if found, might help to elucidate our understanding of the underlying representational competence linking language and play. Data were collected in a sample of approximately 90 families, divided equally among four groups: hearing mothers with hearing toddlers; hearing mothers with deaf toddlers; deaf mothers with hearing toddlers; and deaf mothers with deaf toddlers.

Methods Employed:

In the main longitudinal study, eight groups of (approximately) 30 primiparous mothers and their 5-month-old infants were recruited. With the exception of an adolescent sample, all mothers are Caucasian, married women with healthy, term infants. The groups are composed of approximately equal numbers of male and female infants. A group of teenage mothers comparable to the other groups in terms of race, parity, and health status of the infant has been recruited, but there is variation on other dimensions within this group: for example, most of these teenage mothers are unmarried. Appropriate referral sources were used for recruitment (well-baby clinics, adoption agencies and support groups, public school programs for teenage parents, and advertisements in newspapers and other pertinent publications). Volunteers were screened by telephone by trained members of the research staff.

The design of the study at the infancy age-point provides for six sets of comparisons. In each, one major independent variable is the focus of investigation; other independent variables (parental education, socioeconomic class, marital status, family intactness, the baby's antenatal care, circumstances of labor and delivery, birth order, the baby's health since birth, and postnatal alternate caregiving arrangements) that might influence the dependent variables being assessed are accounted for. Set I examines three groups, varying in terms of maternal age: teenage mothers with mean age less than 20 years, younger mothers with mean age between 20 and 30 years, and older mothers with mean age greater than 30 years. Set II is comprised of two groups varying in terms of mothers' employment status: homemaker mothers and mothers employed outside of the home. Set III includes two groups of employed mothers, differing in terms of the type of substitute care experienced by the infant during the mothers' absence: Infants who experience one-on-one care by a single caregiver in their own home and infants who experience care by a single adult in that adult's home with other children present. Set IV includes two groups varying on parenthood status: mothers by birth and mothers by adoption. The group of biological mothers between the ages of 20 and 30 years is common to Sets I, II, and IV. Set V utilizes families varying in socioeconomic status. Set VI involves comparing the firstborn and secondborn infants of the same mothers.

Each mother-infant dyad is visited in the home setting when the baby is approximately 5 months old. At that time, a three-part videotape record of the infant is made. (In the following description, the mother is referenced. For two groups in the study, the baby is also seen with his or her substitute caregiver. The procedure is the same, regardless of the identity of the adult.) For the first hour, the mother is asked to go about her normal routine, and the infant's typical experience when at home with mother is filmed. Second, the infant is filmed alone with seven favored toys for 10 minutes. Finally, a 3-minute film is made of the mother holding the baby on her lap and interacting en face in a natural fashion. All visits are scheduled for a time when mother and infant are typically at home together and the infant is awake and alert. Mothers are encouraged to behave in their usual manner and to disregard the observer's presence insofar as possible. Mothers are also asked to complete a brief set of questions providing information on demographic characteristics of mother, father, infant, and substitute caregiver (for those caregivers being observed), the baby's experience with substitute care, as well as aspects of the baby's birth or adoption. The videotapes are coded by trained personnel using a behavioral system originally developed in connection with previous work in this area by the Principal Investigator and revised, expanded, and computerized for the current project. Throughout the course of the study, 20% of the tapes are independently coded by two observers for purposes of assessing interobserver reliability.

Each mother and toddler dyad is visited again at home when the child is 20 months old. During the single 2-hour visit, the following data are gathered: measures of the child's play while she or he is playing alone and mother-child interaction during a joint play session; three measures of the child's language development (one obtained from the mother, the second assessed independently by the experimenter, and the third based on transcribed conversation between mother and child); a measure of maternal intelligence; and updated information about mother's employment and concomitant substitute care for the child that have occurred since the baby was 5 months of age. At the completion of the visit, the following domains are assessed: mothers' perceptions of the parental role, mothers' knowledge of various aspects of infant development, and aspects of maternal personality. Finally, information about the child's level of functioning with respect to communication skills, daily living skills, social responsiveness, and motor development is obtained.

At the third data point in the longitudinal study, the child is seen at 48 months of age in the laboratory playroom for one 3-hour visit. Data of various kinds are gathered from the child, including the *Wechsler Preschool and Primary Scale of Intelligence-Revised* (WPPSI-R), a standardized measure of verbal and performance abilities related to intelligence in young children.

In the first ancillary study, an independent sample of 32 children (16 males, 16 females) was recruited. Selection criteria included: Normal pregnancy/delivery status, intact families who are native U.S. citizens and are English-only speakers. At 24 and 36 months of age, the following data were collected: Language and play (at each age, naturalistic samples of each child's language and play have been audio- and videorecorded in two sessions, one session in the home and one in the laboratory), other measures of language performance, structured play, maternal intelligence, maternal perceptions of child temperament, and measures of maternal knowledge of infant development and perception of parenting behavior. At 48 months, the children were given the WPPSI-R. At 48 months, measures of each child's "theory of mind" were also obtained. The children were questioned about the actions of characters in stories. Six tasks were used: (1) a "false belief task" which assessed the child's understanding that individuals can have beliefs or thoughts that do not correspond to reality; (2) a "relative certainty of mental verbs task" which assessed the child's understanding of the relative certainty of mental verbs in relation to external reality (e.g., that the mental verb "know" expresses more certainty than the mental verb "think") in the context of a hiding game with puppets; (3) a "pretense as mental representation task" which assessed the child's understanding that pretending necessarily involves a mental representation as opposed to involving only action; (4) a "pantomime comprehension task" which assessed the child's ability to comprehend actions with imagined objects; (5) a “referential communication task" which assessed the child's understanding of the effects of ambiguous and informative messages on their own knowledge state within the context of a game; and (6) an "understanding of emotions task" which assessed the child's understanding of emotions and feeling states. Data collection is complete in this study.

In the study of deafness and children's mental abilities, children were recruited with the help of schools for the deaf in Delaware, Maryland, New Jersey, Pennsylvania, Virginia, and West Virginia, as well as through newspaper advertisements. Groups were matched for child's age, socioeconomic status, parity, and gender, with deafness being the major disability involved. Each dyad was visited for approximately 2 hours in the home by two researchers and, when necessary, a deaf interpreter. The following data were gathered: videotapes of two 10-minute naturalistic play sessions, one with the child playing alone with a standard set of toys and one with the child and mother playing with toys; an experimenter-administered "scaffolding" task in which the experimenter elicited increasingly sophisticated levels of play from the child; two measures of the child's language development (one obtained from the mother and the second assessed independently by the experimenter); a measure of maternal intelligence; and descriptive information about the family. At the completion of the visit, the following areas were assessed: mothers' perceptions of the parental role, mothers' knowledge of various aspects of infant development, aspects of maternal personality, and sources of maternal stress. Mothers were asked to provide information about the child's typical play behavior by completing a play inventory and an activity diary. Finally, information about the child's level of functioning with respect to communication skills, daily living skills, social responsiveness, and motor development were obtained. In this study, data collection is also complete, and data analysis has begun.

Major Findings:

In a given year, approximately 4 million new babies are born in the U.S.A., and worldwide each day more than three-quarters of a million adults experience the challenges and rewards of becoming new parents. Nothing rivets the attention or stirs the emotions of adults more than the birth of a child. By their very coming into existence, infants forever alter the sleeping, eating, and working habits of their parents; they change who parents are and how parents define themselves. Infants keep parents up late into the night or cause them to abandon late nights to accommodate early waking; they require parents to give up a rewarding career to care for them or to take a second job to support them; they lead parents to make new circles of friends with others in similar situations and sometimes cause parents to lose or abandon old friends who are not. Parenting an infant is a 168-hour-a-week job, whether by the parent or by a surrogate, because the human infant is totally dependent on parents for survival.

A multivolume *Handbook* was organized and published on diverse and comprehensive aspects of parenting. These include the biology and ecology of parenting, the status and social conditions of parenting, and applied and practical aspects of parenting.

Longitudinal Study--5 Months.

Infancy defines the period of life between birth and the emergence of language approximately one-and-one-half to two years into childhood. Although infancy encompasses only a small fraction of the average person's life expectancy, it is a period highly attended to and invested in by parents all over the world. Parenting responsibilities are greatest during infancy, when the child is most dependent on caregiving and the ability to cope alone is minimal; infants engender responsibility, and they make undeniable demands. Reciprocally, infants may profit most from parental care. Infancy is the phase of the life cycle when adult caregiving is thought to exert extremely salient influences. Not only does caregiving occur at its most intense levels then, but infants are thought to be particularly susceptible and responsive to external events. It is the particular and continuing task of parents to enculturate children, that is to prepare them for the physical, economic, and psychosocial situations that are characteristic of the culture in which they are to survive and thrive. Parents everywhere appear highly motivated to carry out this task, and infants are likewise invested in their parents. At their best, parent and infant activities are characterized by intricate patterns of synchronous interactions and sensitive mutual understandings.

In one chapter of the *Handbook*, the salient features of parenting infants were presented. A brief history of interest in parenting infants was reviewed, followed by a discussion of the theoretical significance attached to parenting infants. Characteristics of infants and infant development that are especially meaningful for parenting were described, including developmental changes in state, stature and physical abilities, perceiving, thinking, acquiring language, expressing emotions, and interacting socially. Principles of parenting infants were reviewed, including direct and indirect effects, parenting behaviors and beliefs, stylistic differences between mothers and fathers, and the mechanisms of action of these parenting principles. Forces that shape parenting during infancy were outlined, including biology, personality, infant effects, and various social, socioeconomic, and cultural determinants. Finally, nonparental (i.e., sibling, familial, and nonfamilial) infant caregiving was described.

Mental growth in the human child consists of the increasing coordination of mind and reality, that is, the abilities to assimilate information in the environment and accommodate the mind to it. Another integrative chapter on infancy provided an overview of infancy studies that bear on the growth of rudimentary processes associated with assimilation and accommodation -- sensing, perceiving, and cognizing -- especially matters involved in stability of mental development from infancy. Some *methods* and *measures* of studying elementary cognitions in young infants were reviewed, with a focus on habituation, the decline in infant attention to stimulation that is increasingly familiar. Arguments for the interpretation of habituation as information processing and as an indicator of infant cognitive functioning were assessed on sundry bases, including notably the predictive validity of habituation for childhood cognitive performance. *Models* of stability and continuity, and of the roles of experience in understanding cognitive development from infancy, especially with reference to habituation were discussed, as were antecedents of habituation. Data on some possible *meanings* of habituation in terms of sources of stability in mental growth were also presented.

Relations between maternal education and indices of infant information-processing performance were examined in a separate empirical study. Ninety-four 3-month-olds were seen in an infant-control habituation procedure. No significant differences in habituation performance by maternal education level emerged. This finding suggests that information-processing indices obtained in the first six months are useful as predictive measures of mental development that are uncorrelated with important markers of infants' environmental rearing conditions.

In another short-term longitudinal study of infant attention, models of stability and continuity in infant development were examined and illustrated. Four approaches to quantifying infant attention were described: habituation, recovery, duration, and brief exposure. With respect to stability and continuity, four possible patterns of development obtain: stability and continuity, stability and discontinuity, instability and continuity, and instability and discontinuity. Results indicated that the instability-and-discontinuity pattern is the most prominent description of the development of attention in infancy, but all four possible patterns of development were in evidence, even in the first months of life and even within a single domain of infant performance.

Longitudinal and cross-sectional samples of infants were also habituated to three different visual stimuli and given

post-habituation tests at birth, and at 1, 2, 3, and 4 months in another study of attention patterns. Three major findings emerged: (1) for two stimuli there was a marked increase in habituation times at 2 months; (2) there were consistent stimulus differences in habituation times which maintained across age; and (3) for two of the stimuli there were changes in post-habituation preferences across age. The findings gave a clear indication that visual development in early infancy is characterised both by change and by continuity. The implications of the findings for understanding perception and attention in early infancy were evaluated.
A computer-based system for the continuous coding of behavioral records developed within our laboratory was adapted in a number of ways for use with various data bases. With respect to the 5-month data, codes were grouped into 15 sets of measures which assess infant behavior (e.g., level of alertness, focus of visual attention, type of tactile exploration, facial affect, type of vocalization, and level of gross motor functioning), maternal behavior (e.g., vocalization, social play, mediation of infant attention to the social and inanimate environment, and encouragement of gross motor control), and aspects of the infant's naturalistic environment (e.g., behavioral context, number and variety of physical locations in which the infant spends time, stimulus characteristics of the visual and auditory environments including detailed characteristics of play materials). Each 60-minute videotape of naturally-occurring mother-infant interaction was coded 11 times. The data that were thereby generated represent a combination of continuously coded measures, time-sampled frequencies, and ratings. Most of this coding has been completed at 5 months in the longitudinal study.

For example, analysis of the continuously-coded behavioral data at 5 months has been undertaken for 37 adoptive mother-infant dyads and a comparison group of 37 birth dyads. Subjects in the two groups were individually paired with respect to infant gender, mother's age and level of education, mother's occupation and hours of employment, and family socioeconomic status. Results indicated no major differences in either infant or maternal behavior in the naturalistic home setting. This represents an important contribution to the emerging literature concerned with parenting and children's development in non-clinical samples of adoptive families. Previous research had clearly shown that adopted children are at risk for a range of learning and behavioral difficulties, but the careful studies needed to pinpoint etiological factors remained to be done. Our analyses represent the first in-depth examination of mother-infant interaction in this population and suggest that, in important ways, the early formative experiences within adoptive families parallel those in families by birth. Analyses of our data for these two groups of families at 20 months and 4 years is currently underway. Clearly, the opportunity to trace the course of development longitudinally for these families is valuable.

Relevant to the organization and understanding of the 5-month findings, several theoretical treatments of infancy, infant development, and the parenting of infants were published in addition to the *Handbook* mentioned above.

Longitudinal Study--20 Months.

In one study, we examined multiple antecedents of variation in mothers' self-reported competence in parenting young children. Relations among sociodemographic and personological characteristics, parenting characteristics, as well as child gender and language and social competence were evaluated simultaneously in a sample of 105 mothers and their 2-year-olds. Mothers' knowledge of child development and children's social competence related positively to mothers' feelings of competence, and hours mothers worked per week and dissonance in didactic parenting related negatively. Structural equation modeling supported several unique predictive relations to maternal competence: Mothers who experienced less intrapsychic dissonance between how they actually parent and how they would ideally like to parent, and younger mothers, reported more confidence in their parenting. Higher SES and fewer hours employed were associated with less intrapsychic dissonance and thereby indirectly enhanced mothers' feelings of competence in parenting.

In the first months of life, infants coo and babble, yet by twenty-four months toddlers can speak some grammatically-correct sentences. Language acquisition lies at the nexus of impressive accomplishments in the perceptual, cognitive, and social spheres of development. Some theoreticians have argued that language learning proceeds strictly on the basis of the child's experiences but others have asserted that first language could only be built on innate biological processes. However, language is too rich, unique, and complex a system for the infant simply to learn, just as it is too rich, unique, and complex a system for the newborn simply to know. One integrative chapter described recent research on how infants develop from nonverbal individualists into interactive conversationalists. The chapter included a brief description of norms and individual variation in early language acquisition, a discussion of how infants' experiences complement their capacities by channeling the development of communication toward increasingly effective interactions and integration into the social world, and consideration of production and comprehension in two important and basic systems of language: perception and production of speech sounds and how children begin to acquire referential forms. Human infants are surprisingly well-prepared in each of these realms, possessing both the motivation and the competencies to ensure that they quickly become full linguistic participants. Infants are also afforded every opportunity by their environment to succeed. The origins of communication in infancy are active and constructive and inextricably embedded in the larger context of adult-infant social interaction. Consequently, there exists much opportunity for the interplay of biology and culture in the ontogeny of language.

In a study of early child language, we sytematically examined relations among diverse aspects of child language from different sources using a variety of instruments. A total of 189 20-month-olds and their mothers contributed information about child language comprehension and production; in addition, child language abilities were assessed independently by an experimenter. Correlations of child language measures with socioeconomic status and maternal education were examined, as were correlations of child performance measures with mothers' verbal intelligence, maternal report measures of mothers' tendency to respond in a socially desirable fashion, and experimenter assessments of child social competence. Structural equation modelling supported (1) strong relations among child language measures derived from observations of the child's speech with mother, maternal reports, and experimenter assessments; (2) the causal (temporal) relation between language comprehension and language expression; and (3) the loading of multiple measures of child language from different sources on a single latent construct of language competence.

Sources of individual variation in child language competence have also been examined. Maternal sociodemographic characteristics, personological characteristics, and language, as well as child gender, social competence, and language competence were evaluated simultaneously in 120 20-month-olds and their mothers. At the zero-order level, child gender and social competence related to child language, as did maternal language, personality, attitude toward parenting, knowledge of parenting, and SES. Structural equation modeling supported several direct unique predictive relations: Child gender and social competence predicted child language (with girls higher), and mothers' language predicted child language comprehension. Maternal language itself was positively influenced by SES, maternal intelligence, and mothers' knowledge about parenting. Finally, children's language was influenced indirectly by both sociodemographic and personological characteristics of mothers.

Naturalistic child solitary play and child collaborative play with mother were videorecorded in 141 20-month-olds. Child solitary play, child-initiated and mother-initiated collaborative play with mother, and maternal demonstrations and solicitations of play were then coded into nonsymbolic and symbolic acts using three codes developed in our laboratory. (These coding schemes have also been applied to the videotapes of child, mother, and experimenter play obtained in connection with the methodological and deaf studies.) Analyses were conducted on the sources of individual variation in child pretense (symbolic) play. Family sociodemographic characteristics, maternal personological characteristics, and maternal affective and cognitive play behaviors, as well as children's gender, language competence, and play were examined simultaneously. Zero-order relations obtained between child play and, respectively, child gender and language, family SES, and maternal verbal intelligence, personality, physical affection, and play demonstrations and solicitations. Structural equation modelling supported the following unique predictive relations: Child language and mothers' symbolic play positively influenced child collaborative play, and child gender and mothers' verbal intelligence predicted child solitary play. Child gender and mothers' verbal intelligence and physical affection influenced mothers' play and so influenced child collaborative play indirectly. The cognitive advantages of child play and maternal influences on child play were interpreted in an adaptive parenting framework.

Longitudinal Study--48 Months

Data have been collected on 75-80% of the overall sample. An investigation of relations among toddler language development, affect, and preschoolers' representations of affectively-neutral and affectively-laden events was undertaken on a subset of children who were seen at the 48-month followup. These relations are of interest for several reasons: First, affect has been found to play a role in the acquisition of language and of other aspects of language-based representational competence. Second, children begin to use their experiences of routine events to organize their real-world knowledge into event representations by around 2 years of age, concurrent with toddler language development. Third, an event involves individuals interacting with each other to achieve a goal, which implicates interpersonal affect and emotion language in the co-construction of an event and therefore the event's representation. Measures obtained at the time of the 20-month observation included SES, maternal IQ, and measures of toddler language expression and comprehension. Measures obtained at 48-months included the WPPSI-R and preschoolers' verbal reports about an affectively-neutral event (i.e., what happens when you have lunch) and an affectively-laden event (i.e., what happens when you go to the doctor). Preschoolers' reports of an affectively-laden event were more likely to contain affective content than their reports of an affectively-neutral event. In addition, a model of sociodemographic variables and toddlers' language capabilities as predictors of prescshoolers' verbal reports of affectively-neutral and affectively-laden routine events was tested using two hierarchical multiple regression analyses, each controlling for sociodemographic variables known to influence language development (SES, and maternal IQ). These analyses indicated that SES was the sole predictor of the number of acts in children's reports of the affectively-neutral lunch event. However, mothers' reports of their children's language production at 20 months significantly predicted the number of acts reported in the affectively-laden doctor event. Together with analyses indicating that children are significantly more likely to include affective content in their verbal reports of going to the doctor, these results suggest that child affect and language may play important roles in the acquisition, organization, and narration of specific routine events.

Ancillary Studies

How is symbolic play in the young child affected by contexts of person and place? Is symbolic play in young children stable? One methodological study had two related goals: first, to assess language performance and play sophistication in the same children under different conditions and at different ages and, second, to determine thereby the best estimators of individual differences in language and play status. Preliminary results indicated that young children play at approximately the same level with different people (mother and a stranger who behaves much like mother) and at about the same level in different settings (the familiar home versus the unfamiliar laboratory). Nor did mothers differ in their symbolic play between home and laboratory. Child and mother symbolic play also showed significant (medium to large effect size) short-term stability. In the case of symbolic play, cross-contextual generalizations appear warranted.

In this same methodological study, the association between children's language development and other aspects of thinking that require symbolic functioning was examined. Language comprehension in children was measured at 24 and 36 months using the Reynell Developmental Language Scales and was found to be stable across these two ages. At 24 months children were asked to perform actions with imaginary objects. Language development is thought to be associated with other aspects of behavior that require representational capacity, like symbolic gesture. We explored associations between multiple measures of language and gesture across the third and fourth years of life. Results indicated stability in language, partial stability in gesture, and consistent associations between language and gesture that were independent of general intelligence. We further explored language-gesture relations by measuring more closely which aspects of language might be related to the symbolic representation of actions with objects. These findings supported the notion that symbolic thought underlies competence in both domains.

Most studies of mothers who have children with disabilities tend to present mothers of children with disabilities as a homogeneous group with a distinct interaction style, rather than individuals whose styles vary from maternal behaviors of mothers of normally developing children depending on many circumstances. Our study was organized to document the influences of the loss of a major communication channel (deafness) on mother-child interaction and on children's developing language and play performance during the second year of life in order to explore the role of children's early language learning environments on the development of symbolic activity. Three measures of child language competency were obtained. Two of the three language measures were obtained by a researcher experienced in dealing with deaf children (the Reynell); the third, a word count, was reported by mothers. All three language variables exhibited similar patterns: The two groups of hearing children (with hearing mothers and with deaf mothers) had the highest means and were nearly indistinguishable, the deaf children of hearing mothers had the lowest mean for each language variable, and the deaf children of deaf mothers fell between these extremes. Deaf children's language delays, unlike those of most other groups of children with disabilities, usually occur because of lack of access to a language model and are not necessarily associated with neurological or cognitive developmental difficulties. Not surprising, deaf children with hearing parents had high rates of language delays and deficiencies. By contrast, no overall group differences emerged for frequency of symbolic play in either the solitary or collaborative play conditions.

Significance for Biomedical Research and the Program of the Institute:

This research is intended to contribute to a basic understanding of factors that shape the normative development of cognitive and social competencies in infants and young children. The data collected at each time period permit in-depth examination of contemporaneous influences on emerging capabilities, whereas the longitudinal aspects of the project provide an invaluable opportunity to trace the developmental course of those competencies. Method development has emerged as an important aspect of this project: Central questions with respect to measurement are being addressed, and innovative computer-based tools for data coding and analysis have been developed and are being implemented. A unique aspect of the longitudinal study is the emphasis on a broad range of outcome measures in the cognitive and social spheres at the preschool-age period. Because traditional psychometric measures of intelligence in the preschool period typically sample from a narrow range of mental capabilities, such measures are limited in terms of the information they provide about the possible impact of antecedent variables. They are also restricted in terms of the outcome variables they successfully predict. This investigation focuses on the "whole child" and strives toward a better understanding of the complex, multidimensional, interdependent nature of growth and development in the human child. Last, the laboratory's work is probing a number of issues which have been identified by the NIH as critical problems with respect to the overall health and well-being of American women and children; these include, among others, parenting among adolescent mothers; the effects upon children of maternal employment and various alternative forms of substitute care; and developmental sequelae for children whose lives are touched by deafness.

Proposed Course of Project:

Complete data have been obtained for nearly all of the 5-month groups in the longitudinal sample. Similarly, most mother-toddler dyads have been revisited at 20 months. Approximately 80% of the children have been revisited at 48 months of age. A follow-up when the children are 9 years old is in the planning stages.

Publications:

Andreassen C, Bornstein MH. An invitation to children's storytelling. Review of Relating Events in Narrative: a Crosslinguistic Developmental Study by RA Berman, DI Slobin. Contemporary Psychology 1996;

Bornstein MH. ed. Handbook of Parenting.
Volume 1: Children and Parenting.
Volume 2: Biology and Ecology of Parenting.
Volume 3: Status and Social Conditions of Parenting.
Volume 4: Applied and Practical Parenting.
Mahwah, NJ: Lawrence Erlbaum Associates, 1995.

Bornstein MH. Pomiędzy opiekunami a ich potomstwem: dwa rodzaje interakcji I ich konsekwencje dlarozwoju poznawczego. In: Brzezińskiej A, Lutomskiego G, Smykowskiego B, eds. Dziecko Wśród Rówieśników I Dorosłych [The Child in the World of People and Objects]. Poznań, Poland: Zysk i S-ka Wydawnictwo, 1995;39-63

Bornstein MH. Parenting infants. In: Bornstein MH, ed. Handbook of Parenting, vol 1. Mahwah, NJ: Lawrence Erlbaum Associates, 1995;3-39.

Bornstein MH. Origins of communication in infancy. In: Velichovsky BM, Rumbaugh DM, eds. Human by Nature: Origins and Destiny of Language. Mahwah, NJ: Lawrence Erlbaum Associates Publishers, 1996;139-72.

Bornstein MH. Stability in mental development from early life: methods, measures, models, meanings, and myths. In: Butterworth GE, Simion F, eds. The development of sensory, motor & cognitive capacities in early infancy: from sensation to cognition. Hove: Erlbaum (UK) Taylor & Francis, 1996;

Bornstein MH. Parenting. In Friedman HS, ed. Encyclopedia of Mental Health. San Diego, CA: Academic Press, 1997;

Bornstein MH. Parenting young children. UNICEF Technical Report, 1996.

Bornstein MH, Bornstein HG. Infancy. In: Bornstein MH, Bornstein HG, Landers C, eds. Infancy and Early Child Development. Florence, Italy: UNICEF Innocenti Centre, 1996;

Bornstein MH, Brown EM, Slater AM. Patterns of stability and continuity in attention across early infancy. J Reprod Inf Psychol 1996;14:195-206.

Bornstein MH, Haynes OM, O'Reilly AW, Painter K. Solitary and collaborative pretense play in early childhood: sources of individual variation in the development of representational competence. Child Develop 1996;

Bornstein MH, Slater A, Brown E, Roberts E, Barrett J. Stability of mental development from infancy to later childhood: three "waves" of research. In: Bremner G, Slater A, Butterworth GE, eds. Infant Development: Recent Advances. Hove, England: Lawrence Erlbaum Associates, 1996;

Lamb ME, Nash A, Teti DM, Bornstein MH. Infancy. In: Lewis M, ed. Child and Adolescent Psychiatry: A

Comprehensive Textbook, 2e. Baltimore: Williams & Wilkins, 1996;241-70.

Mayes LC, Bornstein MH. Infant information-processing performance and maternal education. Early Develop Parent 1995;4:91-6.

Slater AM, Brown E, Mattock A, Bornstein MH. Continuity and change in habituation in the first four months from birth. J Reprod Infant Psychol 1996;14:187-94.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 01120-09 LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Parenting and Infant Activity: A Multiculture Perspective

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. H. Bornstein	Head, CFRS	LCE, NICHD
O. M. Haynes	Statistician	LCE, NICHD
C. Rahn	Research Psychologist	LCE, NICHD

COOPERATING UNITS *(if any)*

ARGENTINA (Berti, deGalperin, Pascual); BELGIUM (Vyt); FRANCE (Pêcheux); ISRAEL (Maital); ITALY (Venuti and Lis); JAPAN (Azuma, Ogino, and Toda); KENYA (Bali)

LAB/BRANCH
Laboratory of Comparative Ethology

SECTION
Section on Child and Family Research

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Experiences in infancy are acknowledged to affect the course and outcome of social and mental development in childhood, and they are credited for some of the distinctiveness of culture. More specifically, cross-cultural developmental studies have historically shown that variations in childrearing styles typically have implications for children's later cognitive performance and interpersonal behavior. Home environments are thought to reflect larger cultural values, beliefs, and customs, and many social theorists have contended that the family generally, and the mother-infant relationship specifically, may be vital to development of the individual and basic to the organization of the culture. As a result, investigators have frequently studied infancy and mother-infant interaction in attempts to address questions about the origins and development of cultural identity. Of course, each society has evolved patterns of childrearing adjusted to its own special demands. For example, it is widely held that Japanese and Americans differ in prominent aspects of their psychological make-ups and that certain social and intellectual distinctions between members of these two cultures arise early in life. Previous study on the nature of infant development in Israeli kibbutzim determined that many decisive aspects of infant care -- particularly the close ties between infant and mother -- vary markedly from the typical American experience. Contemporary France, Italy, and America are relatively similar in terms of industrial level, educational attainment, and living standards, yet these societies differ considerably in terms of history, sociology, and culture. Within-culture comparisons are equally important: Studies of development in Belgium offer answers to the question of how being reared in monolingual versus bilingual environments affects cognition, communication, and socioemotional development. Argentina contrasts middle-class with extremely poor rearing conditions, all in a South American setting virtually unresearched. What differences exist in parenting and in infant activity within and across these cultures? A central purpose of this project is to identify similarities and differences in the childrearing ecologies of Argentine, Belgian, French, Israeli, Italian, Japanese, Kenyan, and U.S. American children.

Project Description:

Objectives:

Parenting is a principal reason why individuals in different cultures are who they are, and are often so different from one another. Adults in different cultures adopt some similar, as well as some different, approaches to parenting. Indeed, at the very heart of the concept of culture is the expectation that different peoples possess different values, beliefs, and motives and behave in different ways. Most contemporary research into parenting is of Western (EuroAmerican) origin. Much less is currently known scientifically about non-EuroAmerican families, children, and parenting. Indeed, to paraphrase one critic, three different cultural limitations have constrained the scope of understanding parenting: a narrow subject data base, a biased sampling of world cultures in its authorship, and a corresponding bias in the audience to which it is addressed. Moreover, for good or ill, researchers in many cultures have been schooled almost exclusively in the framework of Western psychological, sociological, and anthropological orientations. Therefore, many use (if not embrace) similar scientific concepts and paradigms. Of course, projecting essentially Western ideas onto the behavior and experience of peoples developing within essentially different cultural contexts is problematic.

Certain caregiver activities have both immediate and protracted consequences for children's development in the first years of life. Most cross-cultural work investigates childhood using samples of convenience. In this project, research focuses on the extent to which caregiver characteristics associated with particular cultures influence observed relations between maternal interaction patterns on the one hand and the development of children's culture-specific and culture-general social and cognitive competencies on the other. These studies promise to be of theoretical interest because potentially contrasting styles of childrearing can be directly compared while minimizing socioeconomic and educational differences, because of known international differences in children's preparation for school, and because the childcare arrangements in different traditions offer critical perspectives on early human development.

Considerable controversy has surrounded the ramifications of different caregiving arrangements for children's growth, yet few systematic, prospective longitudinal cross-cultural studies of infant and child behavior and adult parenting exist. Specific experiences at specific periods in infancy are thought to exert control over specific aspects of cognitive and social growth. Studies that have explicitly or implicitly adopted this view can be seen to have provided the most useful data on questions of early effects. These findings lead to various lines of research -- one to document similarities and differences in adult-child dyads in different cultural ecologies, and a second to determine the implications of these different ecologies for children's later behavior and performance. This research project as a whole is designed to pursue answers to these questions.

The reasons to conduct cross-cultural developmental research are many. One is description: Insofar as cross-cultural developmental descriptions of structure and process attempt to encompass the widest spectrum of human variation, they are also the most comprehensive in social science. They are vital to delimiting the full range of human experience. In this sense, too, they are critical for establishing valid developmental norms. Our awareness of alternative modes of development also sharpens our perceptions and enhances our understanding of the nature of our own culture. A second reason is explanation: Crossing cultures can provide unique insights in the quest for understanding forces that determine course and outcome in human development. Only the cross-cultural view can expose variables which regulate development, but which may be "invisible" from a monocultural perspective. In particular, this approach can help toward understanding parts which culture-dependent and culture-independent forces might play in the emergence and development of psychological structure and process. Further, cross-cultural study can occasion an unconfounding of variables thought to influence development, but which are normally compromised by monocultural investigation. The third reason is hypothesis testing: Cross-cultural developmental inquiry provides natural tests of special circumstances that might surround development, and it is critical to exploring cultural uniformity versus diversity of psychological and other social scientific constructs. Clearly, many of the reasons that motivate cross-cultural developmental research are descriptive, but salient ones also concern mechanism and process. All these motives at base help to explain adaptation in child development. Major purposes of this project are, therefore, to cross-validate and to extend previous work investigating the influences of caregiver behavior on infant and child development. This project is designed to specify the nature of child-mother-caregiver relations among families living in different normal urban environments and in different atypical environments.

Methods Employed:

This cross-cultural project is fully integrated with the domestic U.S. study as described in Z01 HD 01119-09 LCE, where the protocol is explained in greater detail. Notably, both projects capitalize on many of the same prospective longitudinal designs and measures to evaluate child development and parent behavior across the first 4 years of life. Although the core of the research protocol is constant, some variations have been included in response to culture-specific research interests, as well as requirements of specific societies. Across all collaborations, moreover, cohorts of mothers and babies are being seen (at least) at 5, 20, and 48 months; some are seen at other ages as well.

Most participating cultural groups have been selected to be similar in terms of modernity, urbanity, education, and living standards. In each, the family is typically nuclear in organization; the mother is normally the primary caregiver in the family setting; and parents hold many of the same larger goals for their children, notably social adjustment, educational achievement, and economic security. Therefore, it has been possible to obtain samples from different cultures that are roughly equivalent in sociodemographic terms. At the same time, substantial differences exist among these cultural samples in terms of history, beliefs, and values associated with childrearing. It is important to note that, in conjunction with these cross-cultural analyses, selected and meaningful within-culture comparisons are also being undertaken.

As with the choice of U.S. comparison groups in the domestic project, diverse rationales underpin the selection of particular cultural samples in the cross-cultural project. The reasons to compare and contrast childrearing in Japan and America are compelling. Briefly, contemporary Japan and the U.S. are alike in many sociological respects and, importantly, share many of the same child-centered goals. At the same time, Japanese and Americans are thought to differ markedly in childrearing styles. Japanese and American adults contrast in salient aspects of their psychological make-up, and it is believed that certain of these social and intellectual differences arise early in life. The comparison of Japanese and American mothers in interaction with their preschool-age children is also valuable because of findings concerning possible familial antecedents of differential cognitive development in the two societies. Cross-national studies have shown that Japanese youngsters, before first grade, consistently outperform their American counterparts in academic achievement. This is so even though IQ per se does not apparently differ between children in the two societies. The fact that differences in achievement obtain among preschoolers suggests that forces in the family, perhaps centering around mother-infant interactions, may influence their course. In this comparison, activity and interaction patterns which are distinctive to, as well as those which are similar between, these two disparate cultures are identified, and their predictive validity assessed.

An Israeli sample includes urban families as well as families from *kibbutzim*. On traditional communal kibbutzim in Israel, infants do not live with their parents, but grow up in "infant houses" where they play and eat with age-mates and are cared for by professional caregivers, called *metaplot*. Normally women on these kibbutzim work full time, and the socialization of children is delegated to the community and to multiple caregivers rather than solely to parents. Infants spend time with their parents at random periods during the day and each evening. Because of these circumstances, studies of kibbutz childrearing practices have long been considered instructive for the insights they may provide about the effects of early experience on human development generally, and for the light they may shed on childcare experiences in the U.S. specifically. The kibbutz as a research location permits exploration of provocative questions of differential caregiving effectiveness, because mothers and metaplot typically adopt different balances in basic childrearing by emphasizing, respectively, nurturance and affection versus education and discipline. One factor that makes the Israeli situation unique is the relative equality of social status between parents and childcare workers, a situation quite different from the one in the U.S. where childcare workers are typically of lower education and economic level. Clearly, the kibbutz provides an extreme if not arresting variation of substitute caregiving experiences for children and on this basis constitutes an appealing research setting. Urban-dwelling Israeli nuclear families provide ready (normal) controls and a basis for cross-cultural comparison.

Argentina is a still-developing country plagued by periods of economic instability. During the first part of this study, Argentina experienced hyperinflation -- in July 1989, for example, inflation rose at a rate of four percentage points a day -- with severe consequences for standards of living. Some indicators that reflect this situation included a drop in real wages, rise in the cost of living, decline in citizen confidence, and increase in unemployment -- in all, a disorganization of the social fabric. Such radical cultural variation presented an opportunity to revisit many assumed factors and relations in the ecology of normal child development, such as the generally positive association thought to obtain between socioeconomic status and parenting. One objective of this natural experiment was, therefore, to analyze relations between SES and measures of maternal behaviors and perceptions about childrearing on the one hand and child outcomes on the other. (It seems important to point out as well that one of the major criticisms of contemporary psychology, cited earlier, is its Eurocentrism. As a counterweight, Argentina represents a Latin American, Hispanic culture with sufficient similarity in terms of modernity, education, and so forth to populations studied domestically in North America to permit comparisons, but at the same time a population in which child development is underresearched, if not wholly neglected.)

The nature of communicative, cognitive, and social competence can be specially studied, and unique insights into child language development may be gained, through examination of children's linguistic development in the cognitively challenging situation of the bilingual environment. Although constitutionally one nation, Belgium is in fact two countries in one. Northern Belgium is Flemish in language (ca. 60%), whereas southern Belgium is Walloon French-speaking (ca. 40%). A border exists between the two communities -- although called the "Language Frontier" it is, of course, far more than this. The situation along and above the Belgian Language Frontier makes the study of developmental consequences of exposure to bilingualism a natural one. Most bilingual research focuses on sequential bilingualism in preschool and school-age children (“second language acquisition”); the focus of this research effort falls on simultaneous bilingual experience from infancy. Infant exposure to bilingualism can be defined as a child regularly hearing two or more languages from birth. Earlier views held that children with multilingual heritages were disadvantaged in language development. Today, it is believed that the advantages of bilingualism outweigh the disadvantages in, for example, control of processing and analysis of knowledge. Belgium affords a special experimental setting in which to conduct studies of early bilingualism. Bilingualism in Belgium is inherent to the culture of the country. It is positively promoted; it is also less sequential, less bound to SES, and early developing. Like other samples in this project, the Belgian samples are also middle-class, and so simultaneously permit cross-cultural comparison (and the use of U.S. data as a point of reference for the monolingual Flemish group).

Although parts of one nation since 1861, the North and the South of Italy nevertheless have many historical, cultural, and social differences. From an historical point of view, the South grew under Spanish influence and has maintained an economy and social organization typical of a rural society. Administration is centralized in two big towns (Naples and Palermo) in striking contrast with a majority of small villages. The North of Italy developed under the influence of Central Europe, particularly Austria and France, has prospered on trade and advanced manufacturing techniques, and has developed mostly into industrial cities with autonomous administrations. Our research is being conducted in the Veneto region in the North and in the Basilicata region in the South, in the areas of Padua-Venice and surrounding the village of Ruoti, respectively. This choice represents a classic Italian North-South comparison. These two regions differ importantly in terms of typical family structure, which is strictly patriarchal in the South versus more egalitarian in the North. In contrast with the North, women in the South normally do not work outside the home. There, the education of children is a wholly feminine task, children are rarely separated from their mothers, and fathers and babies are seldom in contact. Moreover, social services and support structures for family and children are nonexistent in the South, and school children are especially disadvantaged. These strong regional differences in the same country suggest a study of parenting in the context of controlled but contrasting family life styles and structures to obtain experimental evidence on the roles of cultural and social factors on parenting and development.

Current status. In Argentina, a total of 95 children and mothers, 59 dyads of middle SES and 36 of low SES, comprise the sample observed in collaboration with Dr. C. Galperín of the Universidad de Belgrano. In addition, 36 children and mothers of low SES have been observed in a rural community outside the interior city of Córdoba in collaboration with Dr. Elinor Berti. In Belgium, 30 monolingual and 26 bilingual mother-child dyads have been observed in collaboration with Dr. André Vyt of the East-Flanders Catholic Institute of Health Care. In France, 40 French mother-child dyads have been observed in collaboration with Dr. M.-G. Pêcheux of the Sorbonne. In Israel, A total of 61 mother-child dyads, 31 living in greater Haifa and 30 living on traditional kibbutzim, have been observed in collaboration with Dr. S. Maital of the University of Haifa. (Children living on kibbutzim were observed on two occasions: In the first, children were seen individually with their primary metapelet; in the second observation, which took place within one week of the first, children were seen individually with their mothers in a similar situation at home. Urban children were observed once, with their mothers.) In Italy, a total of 103 children and mothers (so far 58 from North Italy and 45 from South Italy) have been observed in collaboration with Dr. Paola Venuti of the University of Padua. In Japan, two cohorts have been seen (a total of 48 children living in Tokyo) in collaboration with Dr. M. Ogino of Sophia University, Dr. H. Azuma of Shirayuri College, and Dr. S. Toda of Hokkaido University. In addition, a sample of 30 5-month-old infants and their mothers has been newly recruited in Kenya by Dr. Shashi Bali of Kenyatta University.

Strategy for analyses. Data collection is underway in several of these cross-cultural samples. Planned cross-cultural analyses parallel those being carried out in the U.S. longitudinal sample. Some comparisons address questions of stability, experiential effects, and parenting within cultures. Others examine the origins and development of contrasting cultural styles, notably issues of cross-cultural variation and the nature of coherence in maternal ideas and activities, the composition of maternal responsiveness, emphases of maternal speech, correspondences in mother-child interaction, and styles of mother and child language and play.

Major Findings:

Within-culture comparisons. One intra-cultural study was conducted in Argentina. Specifically, the ideas that Argentine mothers hold about childrearing were investigated, comparing mothers of different socioeconomic levels and urban (Buenos Aires) and rural (Cordoba) residence. Three domains of childrearing were studied: social, didactic, and limit setting. For each domain, mothers' perceptions of their actual and ideal behaviors and of their husbands' actual and ideal behaviors, and mothers' satisfaction with their own and their husbands' behaviors, were analyzed. The results showed significant differences between mothers' ideas about their own and their husbands' behavior, especially in the social and didactic domains of caregiving. Socioeconomic level and place of residence had limited effects on mothers' ideas about parenting.

In an empirical study, a sample of Belgian mothers and their firstborn toddlers was visited at home to observe play behavior and to collect information concerning the child's spontaneous language production, maternal knowledge of child development, a measure of maternal verbal intelligence, and mothers' tendency to answer in a socially desirable fashion. The toddlers were either monolingual in Flemish or bilingual in French and Flemish. The monolingual and bilingual groups did not differ on any demographic or maternal characteristics measures. The toddlers differed in their language production, but not in the proportion of their play which was symbolic. Monolingual toddlers produced more words than bilingual toddlers, even when the bilingual toddlers' words in both languages were counted.

One theoretical paper reviewed hypotheses, models, and concepts that are used in the studies for cognitive outcomes of early bilingual stimulation in children. It focused on variables assumed to mediate between the experience of two different language systems in the period of first language acquisition and the alleged resulting cognitive-linguistic consequences. It is shown that socio-economical background variables, age of onset and quality of organization of bilingual experience, and linguistic characteristics of the languages involved have to be considered in interpreting results of studies of early bilingualism.

The Israeli kibbutz has often served as a model or a point of comparison for childcare and for articulating familial versus extra-familial childrearing. Kibbutz children, like other children with working parents, normally participate in multiple caregiving relationships -- with their mother, with caregivers, and with peers. Historically, communal sleeping arrangements were considered the most salient and distinctive feature of the kibbutz. Today, most children on kibbutz sleep in their family homes. Thus, the contemporary kibbutz children's house has been likened even more closely to daycare. In a theoretical review, we proposed that the unique and shared qualities of kibbutz childrearing are best revealed through an analysis of the kibbutz context as an ecological system. Traditionally on kibbutz, mother, caregiver, peers, and other community members form a relatively close-knit collaborative network vis-à-vis the child. An ecological systems perspective redirects research away from the developmental effects of univariate features of kibbutz and other childcare arrangements and to comparisons based on multivariate conceptions of each context. Thus, concepts from family systems and social network studies, and from multivariate research techniques, are proposed as more suitable for evaluating the developmental implications of contrasting interactive systems such as the kibbutz and other early childrearing environments.

One empirical report directly compared caregiving activities of homemaker mothers from urban nuclear families in Israel with those of employed kibbutz mothers and the professional nonfamilial caregivers (metaplot) with whom mothers share responsibilities for childrearing according to formalized social norms. First, similarities and differences in frequency and sequence of several common parenting activities were assessed in mothers and caregivers. Next, covariation among mothers' and among caregivers' activities are evaluated, and resultant patterns of covariation in these matrices were compared. Infants' exploration and vocalization with adults, and covariation in infant activities, were also studied. Finally, covariation between mothers' and infants', and between caregivers' and infants', activities were analyzed, and patterns of interactions between pairs were compared. The identification and description of activities, interactions, and developmental processes which are similar and different in mothers from urban nuclear families compared with those of kibbutz mothers and professional nonfamilial caregivers, who share responsibilities for childrearing in a formal way, were discussed in relation to developmental issues related to covariation and correspondence of caregiving activity in adult-infant dyads.

Two samples, one from the North and one from the South in Italy, are being studied to explore language and play variation in the children and their mothers. In specific, maternal speech to infants of 13 months in the two intra-cultural contexts was examined to verify how cultural variation influences the contents of that speech. This study sought to analyze and compare the language directed to children in terms of two prominent classes of speech -- affect salient and information salient. Mothers and their firstborn children were individually videotaped in naturalistic free-play interaction at home. Maternal speech was transcribed and coded in terms of the primary function of each utterance. Amounts of affect- and information-salient maternal speech were analyzed in terms of frequency and also transformed into proportions. Analyses of variance (sex and site) and analyses of covariance with SES and mothers' age and educational level were conducted. The results showed similarity in amounts of affect-salient contents of the speech in the two samples: Mothers seem to share feelings and to contribute to emotional exchange via their affect-salient speech to babies. However, differences in the two samples appeared in the contents of information-salient language.

Another analysis examined maternal speech to 13-month-old children in the same two cultural contexts (Padua and Ruoti), with the aim to evaluate if and how intranational cultural variation influences the ways in which mothers use language pragmatically when addressing their children. We hypothesized that mothers in the rural context would adopt speech acts with a control function more than mothers in the urban context, whereas urban mothers would use speech acts with a didactic function more than rural mothers. Primiparous mothers and their 13-month-old children were videotaped at home in a play session. Maternal speech was examined in terms of five different communicative functions (Tutorial, Didactic, Conversational, Control, and Asynchronous) using a coding scheme of 21 categories validated in previous studies. Parents also completed a demographic and living standards questionnaire. The results showed both intracultural similarities in some maternal communicative functions (Tutorial and Asynchronous) and significant intracultural differences in other communicative functions (Didactic and Control). These latter are presumably related to different life environments and childrearing practices, as reported by parents in the questionnaire.

In a third study, we compared 40 dyads of mothers and their 20-month-old children from two of the same different sociocultural contexts, North and South Italy, with respect to the level of play shown in a naturalistic play session. We considered 10 minutes of child solitary play and 10 minutes of child collaborative play with the mother and compared in the two samples the spontaneous activity of play of children and mothers at eight levels of increasing sophistication of play. ANOVAs by sex and site using SES as covariate showed no differences between the two samples of children regarding the frequency and the duration of non-symbolic and symbolic play. Socio-cultural context seems not to influence non-symbolic or symbolic play in the South versus in the North of Italy.

Cross-cultural comparisons. In one project, psychosocial predictors of Argentine and U.S. mothers' decisions to work and amount of hours worked (in working mothers) were examined. U.S. and Argentine primiparous mothers of 5-month-old infants participated. Results revealed that years of marriage and months that mothers worked during pregnancy predict whether or not mothers are employed after childbirth in both settings. By contrast, when the number of hours that mothers worked was analyzed, different results were found in the two countries: Better educated women with higher status occupations work longer hours after childbirth in the United States, whereas better educated women with higher status occupations work fewer hours in Argentina. Different cultural and economic conditions in the two countries appear to mediate psychosocial determinants of women's decision to work.

Activities and interactions of Israeli and U.S. American mothers and their 5-month-old infants were observed in the natural setting of their homes. Similarities and differences in infant visual and tactual exploration and vocalization as well as maternal stimulation and speech between Israeli and U.S. infants and mothers were assessed. Coherence in infant activities and in maternal activities within each society was evaluated, and resultant patterns of coherence between the two societies compared. Last, correspondences between infant and maternal activities in each society were analyzed, and resultant patterns of mother-infant interactions between the two societies compared. Identification and description of activities, interactions, and developmental processes which are similar and different in comparable segments of Israeli and U.S. society were explored, and cross-cultural tests of developmental issues related to coherence and to correspondence of activity in mother-infant dyads evaluated. Israeli and U.S. mothers were found to follow culture-specific paths in striving to meet infants' needs and in achieving socialization goals.

Two studies have examined maternal beliefs in multiple cultural contexts. Central to a concept of culture is the expectation that different peoples possess different ideas and behave in different ways with respect to childrearing. In one study, ideas that Argentine, French, and U.S. American mothers hold about their own and their husbands' actual childrearing, as well as what mothers consider to be ideal childrearing, were investigated in three domains: social, didactic, and limit setting parenting. For each domain, we analyzed mothers' reports of their actual behaviors and of their husbands' actual behaviors; mothers' ideal expectations of their own and their husbands' behaviors; and mothers' dissonance with respect to parenting, i.e., the extent to which mothers' actual and their husbands' actual behaviors each agrees with mothers' ideal expectations for themselves and for their husbands. The results showed consistent parent, country, as well as parent-by-country effects interpretable in terms of overarching cultural beliefs.

In a second study, we investigated beliefs about parenting in Argentine, Belgian, French, Israeli, Italian, Japanese, and U.S. American mothers of 2-year-olds. Mothers evaluated their competence, satisfaction, investment, and role balance in parenting, and attributed successes and failures in parenting to ability, task difficulty, child behaviors, effort, and mood. Systematic country effects for both self-perceptions and attributions emerged and are interpreted in terms of cultural proclivities and emphases. Parents' self-perceptions and attributions help to explain how and why parents behave the way they do in childrearing and provide further insight into the broader cultural contexts of children's development.

Theory:

Certain psychological characteristics arise early in life, and the nature of parent-child interaction is thought to constitute at least one important source of individual development as well as cultural variation. Two theoretical reviews explored formative experiences parents provide young children that relate to the origins and development of contrasting cultural styles through an examination of families from different Western and Eastern cultures.

Understanding of activity and its meaning can be viewed through the prism of context and cultures. A given activity can have the same meaning in different contexts, but one activity can also have different meanings across contexts. Conversely, different activities can have different or similar meanings depending on context. A prime context for discussing relations between activity and meaning -- that is, between form and function -- is culture. In one theory paper, flexible relations between activity and meaning were examined in a unified conceptual scheme, illustrated with phenomena from culture and human development. Both the heuristic value of this perspective and its implications for studies of culture and human development were also considered.

Adults help to shape the cultural lives of children. It is the particular and continuing task of parents and other caregivers to "enculturate" children, that is, to prepare them for the physical, economic, and psychosocial situations that are characteristic of the culture in which they are to survive and thrive. As a consequence, many social theorists have posited that the family generally -- and the mother-infant relationship specifically -- play important roles in the development of the individual, and that they also serve to transmit culture. In an overview chapter, some maternal activities with infants and toddlers thought to constitute central, and perhaps formative, experiences of early life were examined, and how those activities relate to infants' activities as well as to expressions of children's early developing mental and social competencies, like language and play skills, was discussed. The research studies to which this chapter referred address questions of parenting as well as the origins and development of contrasting cultural styles.

Significance of Biomedical Research and the Program of the Institute:

The Section on Child and Family Research was established with the aim of pursuing research on the ways in which children's development is affected by variations in the conditions under which they are reared. Some of these variations are studied in the U.S., where the Section examines aspects of the quality of care, family organization, child temperament, and the like (Z01 HD 01119-09 LCE). In many cases, however, understanding of child development is enhanced by conducting research in cultures outside the U.S., because the variations in rearing conditions are greater and the specific variations are of theoretical importance. Moreover, cross-cultural perspectives help to define norms of human development and help to specify contexts of development and their influences on parents and children more accurately and comprehensively.

Proposed Course of Project:

Data collection and analysis of Argentine, Belgian, French, Israeli, Italian, Japanese, Kenyan, and United States samples continue. Recruitment of additional cultural samples is underway.

Publications:

Bornstein MH. La relazione madre bambino in una prospettiva cross-culturale. Somiglianze e specificità culturali in differenti contesti: Americhe, Giappone, Europa. [Mother-infant relationships in cross-cultural perspective: Similarities and specifics in different cultural settings in the Americas, Japan, and Europe]. In Venuti P, Giusti F, eds. Madre e padre: scienze dell'evoluzione, antropologia e psicologia delle funzioni parentali. Florence, Italy: Giunti Gruppo Editoriale, 1996,119-137.

Bornstein MH. Form and function: Implications for studies of culture and human development. Culture &

Psychol 1995;1:123-37.

Bornstein MH, Maital S, Tal J, Baras R. Mother and infant activity and interaction in Israel and in the United States: a comparative study. Internat J Behav Develop 1995;18:63-82.

Bornstein MH, Tamis-LeMonda CS, Pascual L, Haynes OM, Painter K, Galperín C, Pêcheux M-G. Ideas about parenting in Argentina, France, and the United States. International Journal of Behavioral Development 1996;

Pascual L, Haynes OM, Galperín CZ, Bornstein MH. Psychosocial determinants of whether and how much new mothers work: a study in the United States and Argentina. Journal of Cross-Cultural Psychology 1995; 26:314-30.

Pascual L, Schulthess L, Galperín CZ de, Bornstein MH. Las ideas de las madres sobre la crianza de los hijos en Argentina [Ideas of mothers about child development in Argentina]. Revista Interamericana de Psicologia/Interamerican Journal of Psychology 1995;29:23-38.

Venuti P, Rossi G, Spagnoletti MS, Bornstein MH. Gioco simbolico e non simbolico a 20 mesi: comportamenti di gioco del babino e della madre. Età Evolutiva 1996;

Venuti P, Bornstein MH, Toniatti C, Rossi G. Analisi funzionale del linguaggio materno rivolto a babini di 13 mesi. Giornale Italiano di Psicologia 1996;

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HD 01122-09 LCE

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Longitudinal Assessment of Children's Mental and Social Abilities

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. H. Bornstein	Head, CFRS	LCE, NICHD

COOPERATING UNITS *(if any)*
Infancy Studies Program, New York University (Tamis-LeMonda)

LAB/BRANCH
Laboratory of Comparative Ethology

SECTION
Section on Child and Family Research

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research continues a longitudinal study of mental and social development in two cohorts of children observed repeatedly between 2 months and 4 years of age. The premise of this research is that learning in childhood occurs primarily within an interpersonal context; thus, a central goal is to describe how diverse infant and maternal behaviors relate to the ontogeny of central dimensions of children's mental and social competencies. Children participating in the assessment constitute longitudinal cohorts seen (at least) at 2, 5, 13, 20, and 48 months of age. Major objectives of this research are to study stability, continuity, interaction, and predictive validity in mental and social development over the first years of life.

Project Description:

Objectives:

This project proceeds from the theoretical idea that learning in young children occurs in social contexts. The studies encompassed by the project are designed to explore child, maternal, and family contributions to cognitive and social development in infancy and early childhood by examining a wide range of child abilities and parenting activities. Major objectives of this longitudinal research are the study of stability in child development and assessment of the contributions of infant behavioral styles and early experiences, especially parenting, to later capacities. Two cohorts of mothers and children have participated in longitudinal studies at several points over the first four years of these children's lives. Behavioral, cognitive, social, and temperament variables have been evaluated in children and mothers alike.

Methods Employed:

This research examines interrelations among dimensions of infant cognitive abilities, temperament, behavior, social interaction styles, and mother-infant interaction in two samples, one of approximately 50 mother-child dyads seen when children were 2, 5, 13, 20, and 48 months of age, and the second of approximately 100 mother-child dyads seen when children were 9, 13, 17, and 21 months of age.

Major Findings:

Child Abilities:

Multiple considerations of infancy provide a common framework for many papers. Infancy is remarkable because of the dramatic developmental changes that take place in this short period and because of the influence experiences in infancy can exert on later development. The first years witness significant advances in the shape and capacity of the body and its muscles, substantial growth in complexity of the nervous system, rapid development of sensory and perceptual abilities, major milestones in understanding and mastering the object world, acquisition of basic language skills, formation of significant social bonds, and emergence of characteristic personal and social styles. At no other point in the life span do so many aspects of the individual change so rapidly and so fundamentally. The start of the second year is a period of transition and substantial variability in language, play, and attention abilities. In this year, the research program has continued examination of interrelations among dimensions of infant ability and style and mother-child interaction over 2-, 5-, 13-, and 20-months. Several noteworthy results have emerged from these analyses.

A series of studies examined toddler abilities and maternal parenting activities in relation to the development and expression of second-year abilities. One methodological and substantive study defined a set of common quantitative measures for child and mother language and then evaluated covariations, continuities, stabilities, and concurrent and predictive correspondences among those measures in two contexts (play and mealtime) at two child ages (13 and 20 months). Vocabulary, mean length of utterance, and total complete and intelligible utterances minimally to moderately covaried in both children and their mothers. Children and mothers both grew in all language measures across the second year, but they varied in language level in different contexts of play and mealtime. Children and mothers both also showed significant, if moderate, stability in all three language measures. Relations among specific aspects of language between children and mothers at the same age in the same contexts did not emerge; and more child effects on maternal language between 13 and 20 months emerged than mother effects on child language. In the case of language, cross-age or -context generalizations appear unwarranted.

In a related longitudinal investigation, prediction from children's own first linguistic achievements around 9 months and their mothers' verbal responsiveness at 9 and at 13 months was examined in relation to the developmental onset of three significant language milestones of the second year: vocabulary spurt, combinatorial speech, and language used to express a memory. These analyses utilized survival analysis, a statistical technique well suited to questions concerning when in development certain events begin and the extent to which predictors influence the timing of those events. The timing of children's first words in production, the timing of their achievement of 50 words in receptive language, and maternal responsiveness each contributed uniquely to variation in the timing of the three linguistic milestones. Girls achieved the vocabulary spurt, combinatorial speech, and first expression of a memory earlier than did boys. When child and mother factors were considered together, the onset of the three linguistic milestones differed by as much as 5 months for children in the lower and upper 10th percentiles of the predictor variables. These findings contribute to generating and testing specific models about child and mother factors that explain variation in key aspects of children's second-year language development.

One integrative review chapter concerned children's early vocalizations and language development. The chapter was divided into three main parts. First, longitudinal and cross-cultural measurements of infant vocalizations in the first year were reported; factors that influence infant vocalization explored; and effects of infant vocalization described. The second part reviewed data on stability and change in as well as and mother-child interactional influences on children's language performance in the second year. In the third part, nonlinguistic factors that affect language development from infancy, including infant information-processing abilities, maternal interaction styles, and infant effects were discussed. A consistent theme in this chapter is the significance of individual variation in child and parent for child language study.

Researchers and theoreticians in child development often speculate about the nature and correlates of children's early play. What is play like? How does play change over the course of childhood? What inner abilities might play reflect? One investigation of mother and toddler play had two goals: The first was to describe maternal scaffolding behaviors in play interactions by examining the types of play mothers introduce in response to their toddlers' play, and the second was to examine the role of maternal knowledge about child play in maternal scaffolding. Fifty mothers and their 21-month-old toddlers were observed at home in free play. Three levels of maternal play (i.e., exploratory, nonsymbolic, and symbolic) were coded with respect to toddler play. Sequential analyses addressed the first goal and revealed that mothers adjusted the level of their play to match or advance their children's play level. A second set of analyses revealed that mothers who were more knowledgeable about the development of child play responded to their children's play with more play at higher levels than their children's play. Thus, mothers as a group engage in play with their toddlers in psychologically meaningful ways, and mothers with more knowledge about child play development provide their children with appropriately challenging play interactions.

In addition, two reviews of play and its development were published. In the first, methodological approaches and assessment procedures used in the study of child play were reviewed, and a general model of play developed. In symbolic play, children construct increasingly sophisticated representations of the world as well as relations between symbols and their external referents as they internalize, consolidate, and advance upon their developing cognitions about people, actions, and objects. We suggest that an ecological approach to play, in which cognitive, attentional, motivational, and experiential factors are considered jointly and in interaction with one another, will profitably explain variation among children in this domain of development.

This chapter integrated extant research with our own developmental investigations so as to provide a framework for explaining variation in child play. The chapter began with a brief overview of the changing nature of children's early play. We then reviewed methodological approaches and assessment procedures used in our study of child play. Next, we moved to a discussion of a generic model by documenting background literature that supports the importance of the suggested variables for understanding children's play.

The second theoretical paper reviewed empirical studies that address the role and effects of a more sophisticated partner on children's early symbolic play. Presumably, more sophisticated partners, like parents, promote children's development in this domain of action. Yet, the empirical literature to date shows little support for the notion that child symbolic play grows through adult-child interaction. Then presented are three theoretical perspectives that support a view that symbolic interactions with adults ought to promote children's symbolic play and advance children's representational competencies more broadly: They include attachment, scaffolding, and ethological theory. Finally, the paper revisits the literature on interactive influences on children's play reconsidering the nature and role of specific independent and dependent variables in studies of the growth of children's symbolic play.

Parenting:

Parents create people. It is the principal and continuing task of parents in each generation to prepare children of the next generation for the physical, economic, and psychosocial situations in which they must survive and thrive. Many factors influence the development of children, but parents are the "final common pathway" to childhood oversight and caregiving, development and stature, adjustment and success. Early childhood is the phase of the life cycle when parenting is believed to exert its most salient influences: Not only is the sheer amount of interaction between parent and child greatest then, but young children may be particularly susceptible and responsive to parent-provided experiences. Indeed, the opportunity of enhanced parental influence and prolonged childhood learning is thought to constitute the evolutionary reason for the extended duration of human childhood.

The purpose of one paper published this year was to supply a perspective that promotes parental investment in childrearing. The brief is made that it should be the obligation as much as the desire of social institutions to work toward helping parents establish and understand their responsibilities, commitments, and competencies, and institutions should ally themselves with parents to forge support networks, partnerships, and the like to the betterment of children. The essay undertakes a brief review of several key topics in parenting young children. They include the positives of parenting for parents, the purview of parenting, who parents, persistent versus protean aspects of parenting, prerequisites for parenting, present-day problems of parenting, as well as parenting programmes. The essay is concerned less with child outcomes of parenting, and more with the nature and dimensions of parenting per se, conditions of parenting, and recurrent themes in parenting young children.

Theoretical and review papers on mother-child interaction and responsiveness, as well as stability and continuity in development, also appeared in connection with this research. How caregivers respond to infant signals and development, and what consequences, if any, caregiver responsiveness has for the child are issues that were examined in a separate theoretical treatment. First, what responsiveness is and what its typical characteristics were defined. Next, what effects responsiveness has on infant crying and what short- and long-term effects it has on infant cognitive development were examined. Then, how responsiveness operates in growth, and where responsiveness in the caregiver comes from were discussed. Many research reports converge in showing the powerful role that parental responsiveness plays in the development of a broad range of child competencies in development. To provide this kind of responsiveness, caregivers need to know each child in their care, and they can achieve this understanding through developing a close relationship and being attentive and receptive to each child's signals. Responsiveness is a deceptively uncomplicated scenario. Hidden, however, are the telling and powerful consequences responsiveness portends, not only for the child's survival, but for the positive development of intellectual capability and social self-confidence in the child.

In an empirical examination of some of these propositions, specific predictive relations of mothers' responsiveness to their 5-month-olds' nondistress activities and vocal distress for infants' attention span, symbolic play, and language comprehension at 13 months were examined in 36 dyads in a short-term prospective longitudinal study. Maternal responsiveness to infant nondistress activities at 5 months, but not responsiveness to infant distress, uniquely predicted infant attention span and symbolic play, but not infant language comprehension. Mothers' responsiveness at 13 months was positively and consistently, but not significantly, associated with all three infant abilities. The results support the view that the effects of maternal responsiveness on infant mental development are specific and indirect, rather than generic and direct, and recommend further differentiation of infant activity, maternal responsiveness, and child outcome in studies of mental development.

A related longitudinal investigation examined covariation among specific maternal behaviors and their differential prediction of children's language comprehension across the transition to beginning speech. Forty mother-infant dyads were videotaped during free play in their homes when children were 9 and 13 months old. At each age, six maternal behaviors and children's language comprehension were assessed. Two factors of maternal interaction, labeled verbal sensitivity and verbal intrusiveness, were extracted at the two ages, and the two were stable across the 4-month period. Nine-month maternal sensitivity, but not intrusiveness, uniquely predicted 13-month child language and did so over and above children's 9-month language comprehension, which was itself stable between the two ages. Maternal sensitivity was especially influential in promoting comprehension among children who were initially lower in language comprehension, a result that has implications for the design of intervention strategies. These findings confirm models of specificity in parenting by contrasting the importance of maternal sensitivity for children's language development with other kinds of maternal interaction.

In a final study, 64 mothers of children (ranging in age from 6 to 58 months) were tested for their knowledge about language and play using a paired-comparisons procedure in which they were asked to determine, for pairings of play and language items, respectively, which item was more advanced developmentally (e.g., tactually examining an object vs. pretending to feed a doll; saying "bye-bye" vs. saying “I'mommy hat"). This procedure was repeated within a two-week period so that the short-term stability of maternal knowledge could also be assessed. Play items included exploratory behaviors, nonsymbolic acts, and symbolic acts. Language items included prelinguistic communications, single-word utterances, and multi-word utterances of varying sophistication. In general, mothers' orderings of play and language items matched those established in the developmental literature and were stable over the short-term. Mothers' knowledge about language progression was unrelated to and stronger than their knowledge about play, suggesting that parental awareness children's development is domain specific. Lastly, judgements about the developmental timing of both play and language depended on children's current developmental stages. Mothers were less accurate at estimating the timing of milestones that their children had mastered many months earlier, supporting the notion that mothers' sensitivity to play and language is largely informed by children's recent achievements in specific areas.

Significance for Biomedical Research and the Program of the Institute:

The scientific yield of longitudinal assessments of child development and parenting processes holds great potential in itself as well as being tailored to the special needs of future NICHD research projects. In addition, the longitudinal nature of these projects is spelling out critical aspects of parenting involved in the early development of mental and social functioning in children's first years of life.

Proposed Course of Project:

Analyses of these data are ongoing. First, the degree of independence/interrelatedness among child cognitive and social measures is being determined in conjunction with the evaluation of the independence/interrelatedness of these measures with other data sets. This is an iterative process -- results are reviewed and new approaches proposed which, in turn, are submitted to additional evaluations -- and substantial work investment and judgment are required to arrive at a proper understanding of the findings. Predictive relations between observations of these dyads during the first years of life and performance at 4 years are planned. Analyses of the implications of first- and second-year experiences for later preschool development continue.

Publications:

Baumwell L, Tamis-LeMonda C S, Bornstein MH. Styles of maternal interaction and toddler language comprehension: The importance of maternal sensitivity. Inf Behav Develop 1996;

Bornstein MH. Parenting young children. In: Dalais C, Landers C eds. Child in Focus. New York: UNICEF, 1996.

Bornstein MH., Bornstein HG. Caregivers' responsiveness and cognitive development in infants and toddlers: Theory and research. In: Mangione PL, ed. Infant/Toddler Caregiving: A Guide to Cognitive Development and Learning. Sacramento, CA: California Department of Education's Bureau of Publications, 1995;12-21.

Bornstein MH, Bornstein HG, Landers C. Infancy and Early Child Development. Florence, Italy: UNICEF Innocenti Centre, 1996.

Bornstein MH, Tamis-LeMonda CS. Parent-child symbolic play: Three theories in search of an effect. Develop Rev 1995; 15:382-400.

Bornstein MH, Tamis-LeMonda CS. Des vocalisations prélinguistiques à la communication linguistique: facteurs langagiers et non langagiers. [Language and nonlanguage factors in development of prelinguistic vocalization to linguistic communication.] In: Konopczynski G, Vinter S, eds. Le Développment Langagier: Une Prédiction Précoce Est-elle Possible? [Language Development: Is Early Prediction Posible?]. . Calais, France: Ortho-Edition, 1995;81-102.

Damast AM, Tamis-LeMonda CS, Bornstein MH. Mother-child play: Sequential interactions and the relation between maternal beliefs and behaviors. Child Develop 1996;

Tamis-LeMonda CS, Bornstein MH. Variation in children's exploratory, nonsymbolic, and symbolic play:

An explanatory multidimensional framework. In: Rovee-Collier CR, ed. Advances in Infancy Research, vol 10. Norwood, NJ: Ablex, 1996;37-78.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01123-06 LCE

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Physiological Correlates and Neural Mechanisms of the Infant Cry & Related Vocalizing

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	John D. Newman	Section Head	LCE:NICHD
Other:	Kathleen Moody	IRTA Fellow	LCE:NICHD
	Janet Norcross	IRTA Fellow	LCE:NICHD
	Kurt Hammerschmidt	Fogarty Fellow	LCE:NICHD
	Bernhards, Deborah	Bio. Lab. Tech	LCE:NICHD

COOPERATING UNITS *(if any)*

none

LAB/BRANCH

Laboratory of Comparative Ethology

SECTION

Developmental Neuroethology Section

INSTITUTE AND LOCATION

NICHD, NIH, Poolesville, Maryland 20837

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project currently focuses on gender differences in primate vocal communication in adulthood and during development, on the role of individual experience in the acquisition of adult vocal skills, and on the development of computer-based algorithms for acoustic parameter extraction and analysis. Major findings this year were:

1. The vocalizations of aged humans suffering from various forms of dementia are characteristic of dementia subtype. Analysis of acoustic structural details indicates that the vocalizations of subjects diagnosed with Alzheimer's dementia, vascular dementia and dementia of unknown origin are distinctive, such that a discriminant function analysis correctly predicts dementia subtype in about 90% of cases.
2. The vocalizations of developing rhesus macaques recorded during brief periods of social separation are individually distinctive from the newborn period and, with maturation, become more stereotyped and converge in a subset of acoustic parameters. However, other acoustic parameters remain distinctive for the individual vocalizer over this same period.

Project Description:

Objectives:

The major goal of this project is to understand the neurophysiological and neurochemical mechanisms and related brain substrates underlying the expression of vocalizations in nonhuman primates and the relationship of these mechanisms to those regulating the vocal expression of affect in humans. The present investigations use vocalizations as behavioral probes for assessing the potential clinical value of novel pharmacological agents, for assessing the behavioral effects of experimental neurological procedures, and for examining the correlation between vocal and nonvocal measures of emotional state.

Methods Employed:

Standardized testing paradigms involve the natural vocal response to social separation in squirrel monkeys (Saimiri), rhesus macaques (Macaca mulatta), and common marmosets (Callithrix jacchus), as well as the vocal alarm exhibited to threatening external stimuli. Quantitative, computer-assisted analyses of the production rate and acoustic structure of vocalizations in these tests provide objective assessments of subject response during experimental and control trials. Computer-based methods are used to identify specific vocalizations according to established diagnostic criteria and to measure acoustic differences related to experimental procedures. Neuroendocrine and neuropharmacological techniques involve behavioral assessments following administration of systemic and intracranial doses of peptide and nonpeptide hormones and drugs with activity at specific neurochemical receptor sites. Brain sites of peptide and drug action are determined using autoradiographic and immunocytochemical techniques.

Major Findings:

A. Autoradiographic demonstration of brain receptors for oxytocin in the common marmoset and squirrel monkey.

Receptor autoradiography studies of common marmoset brains have continued this year, adding additional male and female brains and using ligands for central oxytocin and vasopressin receptors to compete with 125I-labeled ligand selective for oxytocin receptors ($([^{125}I](d(CH_2)_{5[}Tyr\ (Me)_2\ Tyr\text{-}NH^9]OVT$ or $[^{125}I]OTA$) to determine the ligand specificity of the binding sites revealed by autoradiography. Several binding sites for 125I OTA have been found. The largest and most rostral appears in and around the nucleus accumbens and olfactory tuberculum. Rostrally, this appears as a single large field, but separates into two fields more caudally. This binding site is visible in adult and subadult male and female brains and is relatively specific for oxytocin receptor ligands, as $[^{125}I]$ OTA is displaced by 'cold' vasotocin (a selective oxytocin receptor antagonist) but not by linear AVP (a selective vasopressin receptor agonist). A second, separate binding site, small and very localized, occurs near the nucleus basalis and lateral amygdala. Nissl-stained histology is in progress, to determine more precisely the anatomical locus of this second binding site. The label also appears in the midbrain and caudal brainstem, indicating that there are additional oxytocin binding sites in brain regions caudal to the forebrain, as well.

B. Immunocytochemical Demonstration of Neuropeptide Distribution in the Brain of Common Marmosets.

We have conducted an investigation of oxytocin and vasopressin immunoreactive neurons in the brains of common marmosets, in collaboration with Zouxin Wang and Thomas Insel. With respect to vasopressin immunoreactivity, we have examined the forebrains of 3 males and 3 females ranging in age from 7 months to 4 years. Brain sections were processed for vasopressin immunoreactivity (AVP-ir). The number of AVP-ir cells was counted bilaterally during microscopic examination. A dense cluster of AVP-ir cells was found in the paraventricular nucleus of the hypothalamus and caudally in the periventricular nucleus. Another cluster of AVP-ir cells was found in the supraoptic nucleus. Scattered AVP-ir cells were found in the lateral hypothalamus, suprachiasmatic nucleus, and stria terminalis. This last structure was the only brain area to exhibit sexual dimorphism in number of AVP-ir cells, with males having more cells than females. AVP-ir fibers were found in the hypothalamus, as well as the nucleus accumbens, septum, diagonal band of Broca, lateral preoptic area, and stria terminalis. Another set of brain sections from the same 6 marmosets was processed for oxytocin immunoreactivity (OT-ir). The same procedure as was used for AVP-ir was used for determining number of OT-ir cells in specific brain regions. Scattered OT-ir neurons were found in the same 3 hypothalamic nuclei containing AVP-ir cells,as well as the stria terminalis, medial amygdala, and lateral hypothalamus. No OT-ir cells were found in the suprachiasmatic nucleus. Male and female brains did not differ in the number of OT-ir neurons in any area examined. Finally, brain sections from 2 males and 2 females were processed for androgen receptor immunoreactivity (AR-ir). Cells that stained for AR-ir were found mostly in the cerebral cortex, preoptic area, hypothalamus, lateral septum, amygdala, and bed nucleus of the stria terminalis. Male brain sections had more AR-ir cells in the lateral septum, stria terminalis, and medial amygdala than female sections. An alternate set of brain sections from the same 4 marmosets were processed for both AR-ir and AVP-ir or AR-ir and OT-ir, to determine the presence of co-localization of AR-ir with AVP-ir or OT-ir. There was a lack of AR-ir colocalization with AVP-ir cells in virtually all areas examined. Some AR-ir colocalization with OT-ir was found in the paraventricular nucleus of the hypothalamus. This is the first study of AVP and OT immunoreactivity in a New World primate, and the results show some differences with previous reports in other species. The pattern of AVP-ir distribution in marmosets is different from that reported in macaques as well as in rats, and it may represent a transitional AVP cell distribution between rodents and Old World primates. While a sexual dimorphic pattern in the distribution of AVP-ir cells has been reported in other species, finding it in a monogamous and largely monomorphic species like the marmoset is surprising.

C. Hormonal Correlates of Social Behavior in Squirrel Monkeys

Squirrel monkeys are highly vocal and social New World primates for which there is extensive prior work on vocal communication. One study has suggested that changes in vocal behavior are associated with estrus in Gothic type squirrel monkeys (Smith et al., Folia Primat. 40:233, 1983). In the present study, the relationship between vocal behavior, reproductive hormones and the presence of a male was examined in two groups of Gothic and Roman arch female squirrel monkeys (4 adults, > 7 yr; 4 juveniles, < 3 yr, per group). Introducing an adult male into Gothic female groups, where physical interactions can occur, has been found to initiate female cycling within fourteen days; concurrently, the endogenous testosterone level of the male increases (Mendoza & Mason, Physiol. & Behav. 49:471, 1991). In the present study, the female groups were exposed to a male of the appropriate subtype for 18 days. The males were housed separately, within 0.5 m of the females' enclosure, to prevent pregnancies. Each group was maintained separately. Five-minute focal animal observations were collected under three social conditions: eight observations per animal were collected prior to the introduction of the adult male, twelve observations were collected beginning the day after the male was introduced to each group, and four observations were taken beginning 72 hours after the male's removal. Vocalizations, identified by ear, were scored on-line (the Observer 3.0, Noldus). Blood sampling for hormone assay was conducted frequently on all adults to assess reproductive cyclicity.

A total of 1039 calls were scored (Gothic=587; Roman=452). There were no differences in call rates between species in any condition. Juveniles in both groups showed no changes in vocalization rates or call types. However, adults of both species produced significantly more of an affiliative vocalization, the chuck, than did juvenile females, regardless of male presence (Gothic, adult 4.66 +\-.96/min, juv .32 +\- .14/min, Mann-Whitney z=-3.97, p=<0.0001; Roman, adult 3.86 +\- .39, juv .009 +\- .004, Mann-Whitney z=-4.24, p<0.0001).

Adult Gothic females produced more affiliative vocalizations in the male's presence than in the other two conditions (male presence, 3.48 + 1.32/min; male absence, 1.80 +\- 0.85, Wilcoxon z=-2.37, p=.018). Adult

Gothic females also produced the calls previously associated with the estrus cycle, errs and purrs; these occurred five times more frequently in the presence of the male. However, overall incidence of errs and purrs remained low (male presence, 0.10 +\- 0.05/min; male absence, 0.008 +\- 0.008). In contrast, Roman females did not produce errs or purrs, and increased aggressive calls in the presence of the male (male presence, 0.35 +\- 0.10/min; male absence, 0.12 +\- 0.06, Wilcoxon z=-2.20, p=.028).

Tactile stimulation may be the primary mode of activation of reproductive cyclicity in the female squirrel monkey. Hormonal assays revealed that visual, auditory, and olfactory stimuli are ineffective stimulators of reproductive cyclicity in the adult female squirrel monkey. With the exception of one female, progesterone values were at basal levels throughout the study. One Gothic female had levels indicative of cycling prior to the introduction of the male into the colony room; these levels plummeted to baseline levels upon his introduction. In contrast, testosterone levels of the Gothic male increased from nondetectable levels to 291 ng/ml 19 days after his introduction; the testosterone of the Roman male increased marginally from 7.2 to 8.7 ng/ml.

Although the behavior and vocalizations of the males in this study were not recorded, it is possible that their hormonal states affected their behavior, which, in turn, may have influenced the behavior of the adult females. We conclude that although vocalizations by juvenile females were unaffected, calls of adult females change in the presence of an adult male. The hormonal status of the male may indirectly influence the specific vocal changes observed. Further study will investigate if the production of estrus-related vocalizations (errs/purrs) is faciliated by high endogenous reproductive hormones during cycling.

D. Attachment and Separation Reactions in Common Marmosets

Thirty-three postpubescent marmosets were included in a study of relationships between the social environment, endogenous hormone levels, and behavior. Twelve subjects (6 males, 6 females) housed in family groups were separated from group members and exposed to an unfamiliar, opposite-sex conspecific in a neutral arena. The pair then remained housed together for 24 hours. An additional 9 subjects (4 males, 5 females) were subjected to the same treatment, but placed in the neutral arena by itself for 20 minutes, then returned to their home cages. A third group of 7 males were each separated from group members, placed in a neutral arena for 20 minutes of observation, then kept separated form an additional 24 hours. A fourth group (3 males, 1 female), each singly housed prior to testing, was observed in a neutral arena for 20 minutes then returned to its home cage. For each subject, a 20-minute session in their home cage on the day prior to separation was obtained, followed by 20 minute sessions in the neutral arena on the day of separation, and a third 20-minute session the following day. Femoral blood samples were collected on the day prior to testing, immediately following the 20-minute separation, 30 minutes and 90 minutes following the separation test (after return to their home cage), and 24 hours following the separation. The results indicate that, in those subjects separated for 24 hours, serum cortisol increased significantly by 90 minutes from the start of separation (mean increase of 111.4 per cent), and rose to even higher levels by 24 hours (mean increase of 163.5 per cent). No sex differences were found in these responses. No significant changes in cortisol levels were found in those subjects experiencing only 20 minutes of separation. The vocal behavior of these groups contrasted rather strikingly with their cortisol responses. Subjects from all groups exhibited high levels of calling during the 20-minute separations (pretest mean, 1.17 calls/min; neutral arena mean, 16.0 calls/min). Following 24 hours of separation, calling rates had fallen to 1.57 calls/min, during a time when cortisol values were highest. Vocalizations of animals paired with a new mate remained higher one day after pairing than prior to pairing (pre-pairing, 1.08/min; post-pairing, 2.33/min, z=-2.156, p=0.031), although cortisol levels in these paired animals

Significance to Biomedical Research and the Program of the Institute:

This project has identified brain structures and related neurochemical mechanisms implicated in regulating vocal expression in the contexts of social separation and alarm. The present studies aim at improving our understanding of mechanisms related to the vocal expression of affect in humans, an important but poorly understood aspect of human behavior. Since the principal behavioral probe used in these studies, the primate isolation call, is structurally and functionally homologous to the cry of the human infant, the present investigations provide a comparative approach to studying the infant cry as a marker of normal neurobehavioral development. The addition of neuroanatomical and endocrine techniques to the ongoing studies provides new and important analytical tools for determining the neural substrates and related neuroendocrine and neuropeptide actions that may be involved in directly mediating social behavior in nonhuman primates.

Proposed Course:

(1) Autoradiography and immunocytochemistry of neuropeptides: The completion of a brain map for the distribution of oxytocin binding sites (putative oxytocin-specific receptor sites) in the brain of the common marmoset is anticipated. It will be of interest to examine the changes in the distribution and density of binding sites with the age and infant care-giving experience of the individual, as well as the relationship between gonadal steroid production and synthesis of neuropeptides and their receptors. Given the differing social organization of marmosets and squirrel monkeys, it would eventually be valuable to compare the distribution of oxytocin binding sites and the roles of age, gender and experience in modulating oxytocin production and binding in this species with the information gathered in the marmoset. A useful comparative model based on differing social organization, affiliative bonding, and infant caregiving strategies in two species of vole has already been developed providing a heuristic model for further studies with the nonhuman primates in our colony. (2) Studies of care-giving in marmosets: Based on the data from the juvenile retrieval studies, there appears to be a clear activation of a specific behavior (infant retrieval) within a proscribed time period. We plan to pursue this study by utilizing c-Fos gene activation techniques to determine particular areas of the brain that may be involved in the initiation of retrieval behaviors in previously naive juveniles.

Our plans also include the use of intracerebral microinjections of oxytocin, vasopressin, and prolactin to determine the effect of these neuropeptides on specific behaviors, such as infant retrieval. Our efforts to localize the receptors of interest within the brain should facilitate our intracranial studies. (3) Role of gonadal steroids in mediating the gender differences in cholinergic activation of defensive threat behavior in squirrel monkeys: This role needs to be explored further, given our demonstration that intact females and castrated males show a lower threshold and stronger vocal response to the alarm-enhancing anticholinergic drug, benactyzine HCl. We plan to treat castrated males with daily systemic testosterone over an extending period, assessing the treatment effects on vocal response to a standardized test for eliciting “alarm” behavior. Other studies will test the behavioral effects of temporarily blocking endogenous aromatase activity in intact males and females, since estrogen is the centrally active steroid hormone in both males and females and is synthesized in the brain by aromatase activity.

Publications:

Moody KM, Adler, NT. The role of the uterus and cervix in systemic oxytocin-PGE$_2$ facilitated lordosis behavior. Hormones and Behavior, 1995, 29: 571-580.

Newman JD, Farley MJ. An ethologically based, stimulus and gender-sensitive nonhuman primate model for anxiety. Prog. Neuro-psychopharm. Biol. Psychiatry, 1995;19:677-685.

Newman JD, Bachevalier, J. Neonatal ablations of the amygdala and inferior temporal cortex in rhesus macaques alter vocal response to social separation during the first year of life. Brain Research, in press.

Sams-Dodd F, Newman JD. Effects of administration regime on the psychomimetic properties of d-amphetamine in the squirrel monkey (Saimiri sciureus). Pharmacology, Biochemistry and Behavior, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01124-06 LCE

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Genetic and Experiential Influences on the Development of Primate Vocal Behavior

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	John D. Newman	Section Head	LCE:NICHD
Other:	Moody, Kathleen	IRTA Fellow	LCE:NICHD
	Norcross, Janet	IRTA Fellow	LCE:NICHD
	Hammerschmidt, Kurt	Fogarty Fellow	LCE:NICHD
	Bernhards, Deborah	Bio. Lab. Tech	LCE:NICHD

COOPERATING UNITS (if any)

Emory University (T. Insel; Z. Wang)

LAB/BRANCH
Laboratory of Comparative Ethology

SECTION
Developmental Neuroethology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project is currently focusing on the hormonal, neural and pharmacological factors underlying vocal expression in nonhuman primates. Major findings this year include:

1. An autoradiographic study of the distribution of oxytocin binding sites in the brains of common marmosets found evidence for oxytocin specificity. Sites binding 125I-oxytocin show significant reduction in binding when co-treated with vasotocin (an oxytocin receptor antagonist), but not when co-treated with a ligand for vasopressin receptors. Further localization of forebrain receptor sites was made, with the most prominent encompassing the nucleus accumbens and olfactory tuberculum. A separate area occurs in the nucleus basalis/lateral amygdala region.

2. Hormonal status of single adult male squirrel monkeys living in proximity to a social group of females is correlated with changes in female vocal behavior. Females living adjacent to a male with high circulating testosterone level for 3 weeks showed a 10-fold increase in purrs and errs over pre-male introduction, whereas females living adjacent to a male with low testosterone level did not exhibit any change in their vocal behavior.

Project Description:

Objectives:

The principal objective of this project is to understand the roles of genetic mechanisms and individual experience in the development of normal vocal behavior in primates. Current studies are aimed at identifying developmental stages associated with structural maturation of the isolation call, the emergence and differentiation of vocal categories during development, identification of gender differences in vocal behavior, and the structural and functional relationship between the isolation call and other vocalizations in adult and subadult primates.

Methods Employed:

A comparative approach is used in this project, through the study of a variety of nonhuman primate species as well as human infants. Studies of vocal development involve family or peer group recordings, as well as gently separating the infant from its family and placing it in a recording chamber that provides complete visual separation and partial auditory separation from the rest of the colony. Analysis of vocal data consists first of producing time-histories of the tape-recorded vocalizations with a real-time FFT sound spectrograph, then displaying the analyzed vocalizations on a color video monitor or as hard-copy records for later inspection. Temporal and frequency measures of individual vocal utterances or vocal sequences are made by automated, computer-based parameter extraction methodology, as well as by more traditional hard-copy recordings using a digitizing tablet and microcomputer. In addition to conventional univariate statistical procedures, multivariate procedures including cluster analysis and linear discriminant function analysis are used for characterizing the acoustic parameters characterizing different individuals, age groups, species, populations, neurological status, hormonal status, and behavioral contexts.

Results

A. Effect of social context on phee call production in nonreproductive common marmosets.

Previous work in this lab has shown that the common marmoset phee call exhibits context- and gender-specific attributes, in terms of differences in acoustic structure. As one of the contexts in which phee calls are produced is related to group spacing ('territoriality'), it was of interest to determine the extent to which nonreproductive family members participate in this calling behavior while in their natal group, and whether their vocal behavior changes after removal from the natal group and pairing with a mate. The phee calls of 12 (6 male, 6 female) marmosets (ranging in age between 13.1 months to 20 months at the start of the study) were recorded and analyzed under these 2 social conditions. Periodic samples of phee calls produced during brief social separations were also recorded. After 1 month of study, subjects were removed from their natal groups and housed as heterosexual pairs in new cages. Pairs were made up of animals from different family groups. The effect of changing social conditions was evident with respect to rate of territorial (home cage with partner present) phee calls. In the natal group, only 3 of the 12 subjects called, for a total of 8 phees. In contrast, all 12 subjects made home-cage phee calls after pairing, and calling rates were an average of 25 times higher than in the natal groups. Calling rates of phees made during brief periods of social separation did not differ between the natal and newly paired housing conditions, suggesting that the change in vocal behavior in the home cage context was not merely the result of being older. No sex differences in vocal behavior were observed in this study. These results suggest that the natal environment, consisting of an older breeding pair in addition to varying numbers of sibs, has an inhibiting effect on the home-cage phee calling by nonbreeding family members. The rapidity with which newly paired animals began calling in the home cage condition (as early as 4 days after pairing) suggests that physiological and neuroanatomical substrates necessary for home-cage calling may be in place but remain suppressed until released by the appropriate social context.

B. Vocal behavior of female squirrel monkeys: effect of adult male presence.

A prior study from our lab (Smith, et al., Folia Primat. 40: 233, 1983) demonstrated that squirrel monkeys of the Gothic arch subtype engage in purr-like vocalizations that mirror the 7-11 day estrus cycle in this species when an adult male is a member of their social group. Other studies have shown that the presence of an adult male as a social group member can initiate female cycling within 14 days (Mendoza and Mason, Physiol. & Behav. 49: 471, 1991). A study conducted this year attempted to test whether a male in close proximity to a group of females, but not in the same enclosure, would produce similar changes in vocal behavior and hormonal status In addition, blood sampling provided the means to monitor changes in steroid hormones in these same females. Two female groups, living in separate rooms, were used as subjects. Each group consisted of 4 adults (at least 7 years old) and 4 juveniles (no more than 3 years old). One group was made up of individuals of the Gothic-arch subtype and the other, of individuals of the Roman-arch subtype. After a 3-week period with no male present, a male of the appropriate subtype, previously housed with other males, was introduced to each female group. Each male was housed alone in a full-height cage within 2 m of its respective female group. Behavioral data collection consisted of 5-min. focal observations from each female. Vocalizations were scored by ear and were subsequently validated by tape recording and sonagraphically analyzing some sessions. Blood sampling for steroid hormone assay was conducted 2-3 times/week on each adult female, and just prior to and just after exposure to the females in the 2 males. A total of 1039 calls were scored. There were no differences in calling rates by juveniles of either group associated with the presence of a male. However, adult females of the Gothic-arch group produced significantly more chucks (an affiliative vocalization) in the presence of the male ($p<0.018$). Adult Gothic-arch females also produced purr-like calls 10 times more frequently with the male present. The only change in vocal behavior observed in the Roman-arch adult females was a significant increase in aggressive calls in the presence of the male ($p=0.028$). There was no indication from the hormone assays that any adult female began cycling after male introduction. Only the Gothic-arch male showed a significant increase in testosterone level following the period of close proximity to females.

C. Vocal characteristics of clinically defined subpopulations of aged humans.

In recent years, with the increased lifespan of many human populations, there also has been an increased incidence of clinical cases of dementia and other aging-related mental disorders that need to be understood. In some nursing home settings, residents with varying degrees of mental incompetence produce frequent and disruptive vocalizations which require management to provide a more tranquit environment for the other residents. Such is the case at the Hebrew Home of Greater Washington, in Rockville, Maryland. An opportunity to analyze the acoustic properties of some of the vocalizations of this subpopulation arose through a collaboration with members of the Research Institute of the HHGW, particularly Dr. Jiska Cohen-Mansfield (the director of the RI) and Ms. Perla Werner. This study was of interest because it provided an opportunity to test the efficacy of our computer-based analysis software (LMA, developed for nonhuman primate vocalizations by Dr. Kurt Hammerschmidt) on a novel data set. A subset of 25 nursing home residents (20 female, 5 male), with a mean age of 86.1 years, was chosen for this study. All produced vocalizations repetitively either when alone or in their room or in a larger room with other residents present. In addition, all had been assessed as having dementia, which was categorized as Alzheimer's (16 cases), vascular (5 cases) or of unknown origin (3 cases). A total of 419 vocal utterances, selected from a larger sample, was determined to be of suitable quality for our analysis. Each call was digitized at 12,500 samples/sec, and stored on a hard drive in a Pentium computer. Analysis consisted of first subjecting the digital file to Fourier analysis, using the SIGNAL program (Engineering Design) and a resolution of 1024 points (giving a frequency resolution of 12.2 Hz). Time resolution was set at 25 msec. From each of these FFT files, 85 acoustic parameters were calculated using LMA. A discriminant function analysis (using SPSS

6.1) was run on this data sample, including all 85 parameters, to test for differences in the acoustic properties of vocalizations from individuals with each of the 3 types of dementia. The discriminant analysis correctly classified 91.8 % of the cases, a much higher than chance level of separation into the correct dementia categories on the basis of acoustic differences. Running the same analysis on 50 % of the cases, treating the remaining 50 % of cases as 'unknowns', produced a correct classification of 85.5% of the 'unknown' cases, suggesting that the parameter set had general validity in sorting vocalizations based on the dementia subtype of the vocalizer. This finding is all the more remarkable, given the high degree of individuality in the vocal utterances. One individual might utter drawn-out 'why' sounds, while another individual with the same dementia subtype might utter sounds with a high degree of mouthing. Each subject was highly consistent in the type vocalization used. In those cases where the vocal utterances took the form of intelligible speech, the same words were uttered over and over (such as 'I don't know'). Despite this individuality, there is evidently sufficient sharing of acoustic properties inherent in the vocalizations of each dementia subtype to accurately associate each sound with the dementia subtype of its vocalizer. The potential clinical value of the findings from this study is in the prospect of using the acoustic character of the vocalizations from aged individuals to make a preliminary diagnosis as to the presence of dementia and its possible etiology. It also represents an example of the usefulness of an analytical approach developed for nonhuman primate vocal behavior in assessing clinically relevant aspects of human vocal behavior.

D. Vocal Development in Rhesus Macaques.

A new series of recordings from developing rhesus macaques was made this year, in order to provide a high-quality acoustic database for evaluating and improving a computer-based acoustic analysis system developed for Barbary macaques by Kurt Hammerschmidt (LMA). Vocalizations were recorded during brief separations associated with a developmental assessment procedure based on the Brazelton scale used in human neonates. The assessment and vocal recordings were made by Dr. Maribeth Champoux and Ms. Courtney Shannon, recorded weekly at 1, 2, 3 and 4 weeks of age. Additional vocal data recorded in a previous year by Ms. Susan Jones under similar conditions from older infants (ages 2, 3, 4 and 5 months) were also included. Each vocalization of suitable acoustic quality was digitized using a Pentium computer, then subjected to Fourier analysis using the SIGNAL program (Engineering Design). The FFT files were then used to calculate a large number of acoustic parameters, using LMA. Based on these calculations, a cluster analysis demonstrated that the vocal subtype typically associated with social separation in the rhesus macaque, the “coo,” could be reliably separated from other vocal subtypes produced in the separation context. Approximately 3100 coo calls from34 subjects were then subjected to further analysis, to provide a developmental profile for individual acoustic parameters, and to identify attributes associated with individual differences and the age of the vocalizer. A discriminant function analysis revealed a correct classification of the vocalizations identified by individual vocalizer of 92%, much higher than chance, and indicative of clear differences in the acoustic parameters of coos emitted by different individuals. A similar discriminant analysis was performed on the same data set, this time grouping the calls by age of the vocalizer. The correct classification in this case was only 65 %. Inspection of the classification table suggests that most errors were with adjacent age classes (e.g., 1 and 2 weeks). With respect to changes in individual parameters with age, there was wide variation in the patterns of change. Duration, a very basic parameter (measured from the start to the end of each vocal utterance), failed to show a clear age-related pattern of change, in that the median value remained about the same. However, there was an upward shift in the range of duration values , as expected as the animals became larger and their lung capacity increased. Another basic parameter, mean frequency range, likewise failed to show a clear age-related change, but there was a downward shift in the range, and the variability index (standard deviation) showed a gradual decrease with age. Parameters that did show changes with age included measures of the slope of the fundamental and of the frequency variability of the fundamental. Thus, in general, coos had a flatter slope, less modulation, and a slightly lower pitch with increasing age. These analyses are important, because they provide the basis for future examination of biologically relevant variables, such as type of rearing, individual experience, and measures of temperament or emotionality.

Significance to Biomedical Research and the Program of the Institute:

The respective roles of experiential and genetic mechanisms in the development of normal communication are presently poorly understood. The availability of primate models for studying how individual experience and genetic transmission of species-specific communication patterns interact provides a unique opportunity for analyzing the mechanisms underlying the expression of communicative behavior in primates. The clear homologies between vocal behavior in human and nonhuman primate infants makes a comparative approach to studying vocal development in humans particularly appropriate.

Proposed Course:

1. Vocal ontogeny. This project will continue to devote major emphasis to tracking the developmental changes in vocal behavior. We will attempt to determine the general principles and the variability in developmental milestones, as well as attempt to identify the emergence of gender-specific and individual differences, in each of the primate species under investigation (common marmoset, rhesus macaque, squirrel monkey) and initiate similar studies in a fourth species, the capuchin monkey, . Vocal characteristics will be assessed in genetically related individuals to evaluate the extent of familial traits. Increased emphasis will also be given to the vocal behavior of developing individuals while in their social group. Mother-infant vocal interactions and the differential opportunities to experience adult vocal behavior in young males and females will be studied. The "conversational subsystem" of squirrel monkeys is of particular interest, in that vocalizations used by adult females more frequently engage this communication subsystem than vocalizations used by adult males. The early differentiation of male and female social behavior, including vocalizations, will be studied to identify differences in the maturation rate of vocal behavior in male and female squirrel monkeys. For comparative purposes, the maturation of other vocal subsystems (particularly, the "alarm" and "separation response" subsystems) will also be tracked.

2. Playback studies: We intend to determine whether the interest of family members toward newborn infants is influenced by the vocal behavior ("crying") of the infant, using playback techniques. Conspecific "cries" (recorded from separated infants) will be used, testing for differential responses according to the age, gender, and familiarity of the subject to the source of the playback. We also intend to test response selectivity to subtypes of other vocalizations, particular alarm calls (squirrel monkeys) and recruitment screams (rhesus macaques). Context specificity, familiarity with the source of the vocal stimuli, and (in squirrel monkeys) species differences will be examined. Information gained from these experiments will be useful in stimulus selection for planned studies of the neurophysiological mechanisms underlying species-specific responses to vocalizations.

Publications:

Newman JD, Bernhards DE. The affiliative vocal subsystem of squirrel monkeys. Annals N. Y. Acad. Sciences, in press.

Schrader L, Hammerschmidt, K. Computer-aided analysis of acoustic parameters in animal vocalisations: a multi-parametric approach. Bioacoustics, in press.

LCMB

NICHD Annual Report
October 1, 1995 to September 30, 1996

Laboratory of Theoretical and Physical Biology

This Laboratory studies the elements of biological phenomena to elucidate their physical basis, to provide a foundation for understanding physiological and pathophysiological mechanisms. This Laboratory develops and uses novel, non-invasive technologies to probe physical parameters of living systems and their components. At the same time, the Lab's investigators study systems of well defined molecular composition and structure which exhibit an essential biological function. This approach provides an environment that supports investigation of the physico-chemical basis of molecular, physiological, and pathological processes. Research in the Laboratory includes the biophysics of gas phase organic ions and peptides, polymer organic chemistry for separation of biological macromolecules and particles, membrane biophysics and electrophysiology of ion channels and membrane merger, cellular biophysics of parasite entry and viral infection, and the cellular and tissue basis of human lymphoid tissue function and HIV pathogenesis therein.

MEMBRANE REMODELING DURING VIRAL-INDUCED SYNCYTIA FORMATION AND PARASITIC INVASION OF CELLS

One group, directed by ***Joshua Zimmerberg,*** studies cell surface changes during two pathological events. Membrane topology defines life: the inside of a cell is highly organized at the molecular level, at almost solid-state densities; the outside environment of cells is usually more chaotic. To understand the mechanisms by which cells control their membranes' topologies, they have continued to develop new methods and use new and old techniques to study these two aspects of cell membrane remodeling. The fusion pore is the object through which viruses infect cells; the fission pores is the object through which parasites infect cells. It is essential that they understand these structures at the highest level of resolution in order to design ways of stopping viral infection at the stage of cell entry, and generate novel strategies to arrest infection by parasites.

Theory of admittance measurements. For the Im-based calculation algorithm, a robust measurement of fusing membrane capacitance lags fusion, because the electrical access to new membrane is limited by a fusion pore. As far as G_p becomes negligible compare to an admittance of C_{m2}, an accurate value can be obtained. For their latest, Im/ Re-based algorithm, an accurate value of C_{m2} could be theoretically obtained even for small pores. In practice, when the FP is small, Im/ Re-based values of C_{m2} can still be smaller or higher than expected. They postulated that such deviations are explained by small errors in phase adjustment. C_{m2} can be restored by “off line” correction of : a correct phase angle corresponds to step-like shape of C_{m2}. An option for manual ”off-line” correction of is implemented in their software (“Browse”).

Viral Fusion Pores. To investigate whether other envelope viral proteins or features of a target membrane may interfere with FPs, a fusion of stably transfected, GP64-expressing Sf9 cells with erythrocytes was studied. Eleven GP64-expressing, stably transfected cell lines were produced in collaboration with Dr. Blissard. They bear the OpMNPV strain GP64 (Op GP64). Cell lines were tested for their ability to form fusion pores in the time frame of a patch-clamp experiment; one of them (Op-1D) was suitable for time-resolved admittance measurements. In 66% of experiments (n=53), Op-1D/erythrocyte fusion occurred. Initial fusion pore never flicker; its conductance varied between 0.3-4.6 nS with a median of 1.1 nS. Erythrocyte membrane capacitance was equal to 1.1 0.2 pF (range: 0.8-1.5 pF), close to the expected value. Waiting times from pH delivery to FP appearance varied between 0.16-4.65 s with a median of 0.44 s. Using a Mann-Whitney statistical non-parametric test they compared Op-1D cell/erythrocyte to AcMNPV-infected Sf9 cell/Sf9 cell fusion. Their analysis showed that initial pores induced by two different baculoviruses' fusion proteins had the same initial conductance. Op-1D/erythrocyte fusion had significantly smaller $_{to}$ median value (0.44 *vs.* 0.63 s). For AcMNPV-infected Sf9/Sf9 fusion, the waiting time distribution fit best to a parallel model with many independent elements. The crux of the model is that after triggering, several independent identical elements move from non fusion-permissive to fusion-permissive positions. After all these transitions happen, fusion takes place. This approach is also applicable for Op-1D/erythrocyte fusion: the calculated rate constant of forward reaction is greater then in case of AcMNPV-infected fusion (14.5 *vs.* 6.7 s^{-1}). These results are consistent with the idea that the target membrane and the presence of other envelope viral proteins are unlikely to interfere with the initial pore conductance but rather could have an impact on fusion pore kinetics.

Parasites invade by invaginating the plasma membrane. *T. gondii* is an obligate intracellular parasite. During invasion, which is an active process distinctly different from phagocytosis, the parasite becomes surrounded by the PVM. The PVM pinches off from the host cell membrane at the end of invasion, to create a vacuole within which the parasite grows and replicates to continue the infection. The origin of the PVM in cells is unknown. Two main models have been proposed to explain how the PVM is formed. The bilayer insertion model predicts a significant increase in surface area during PVM formation, followed by a drop in surface area as the PVM pinches off. The induced invagination model predicts no significant increase in surface area during PVM formation, followed by a drop in surface area as the PVM pinches off.. This project is the first application of electrophysiological methods to the study of host cell invasion during parasitic infection.

Parasite gliding stops immediately prior to invasion, when the apical end of the parasite attaches to the host cell plasma membrane. This first attachment leads to a large transient in host cell membrane conductance (spike) (n>100). A constriction, readily visible with DIC optics, then develops at the apical end of the parasite and passes along the length of the parasite as it squeezes into the developing PVM. This constriction is the hallmark of an invading parasite. To independently confirm that a constricting parasite had indeed internalized, they developed an immunofluorescence assay in which free parasites and parasites bound to the external surface of the cell were brightly fluorescent, while internalized parasites were inaccessible to the antibody and were not stained. The capacitance of the host cell did not increase during PVM formation. Two different methods were used to quantitate capacitance changes during PVM formation. By either method, host cell capacitance did not change (0.000 +/- 0.023 pF, s.e.m.; n=11). Since the capacitance change associated with pinching off of the PVM was found to be -0.219 pF, these data are clearly inconsistent (*t*-test, a=.005) with the hypothesis that the PVM is composed primarily of newly inserted bilayer, which would predict an increase in host cell capacitance during invasion of this magnitude. The mean change they measured by both methods of analysis was zero, and the data allow us to rule out (a =.05) any hypothesis in which there is a change of greater than 0.031 pF (method #1) or 0.041 pF (method #2), which corresponds to 14.2% or 18.5% of the total surface area of the PVM, respectively. Capacitance measures the net surface area, and while it is possible that the host cell tightly regulates its surface area and compensates for parasite-added lipids by taking up membrane as lipids are inserted there was no evidence for such regulation after a substantial loss of membrane, the pinching off of the PVM.

The capacitance data show that while a small amount of parasite-derived material (0-18.5 % of the total surface area of the PVM) may be inserted into the host cell membrane during invasion, most, if not all of the PVM is derived from the host cell plasma membrane. Specific proteins and lipids are thought to be transferred from the apical organelles of the parasite to the host cell during invasion, and might serve to induce the invagination or to modify the functional properties of the PVM. Their data are clearly inconsistent, however, with the idea that the PVM is composed primarily of lipids secreted by the parasite. They are also inconsistent with an earlier model in which the parasite enters directly into the host cell cytoplasm by disrupting a region of host plasma membrane, as this model would not predict the PVM-sized drops in host cell capacitance.
They have demonstrated an unexpected delay between the end of constriction and the pinching off of the PVM. This delay occurred even under perforated patch conditions, which minimize cytoplasmic washout. Preliminary experiments using fluorescent antibody against the parasite surface protein P-30 and fluorescent lipophilic probes suggest that prior to pinching off, the PVM is continuous with the host plasma membrane but its interior is inaccessible to extracellular soluble macromolecules.

Parasitic fission pores. Their measurement of the electrical parameters of the cell membrane during the entire process of invasion allowed us to detect the parasite-induced fission pore. A fission pore is the small aqueous connection between a forming endocytic vacuole (here, the parasitophorous vacuole) and the extracellular medium which forms and constricts to closure during the final stages of vacuole pinch off. Detected pore conductances ranged from 12 to 0.1 nS, a range which overlaps with that of molecular ionic channels. The capacitance fluctuations described above reflected a rapid relaxation from a semi-stable pore conductance to a larger conductance, followed by a second (or third) constriction and complete closure. No additional increase in cell conductance, which would indicate a breach in cell integrity, was detected. Compared to membrane fusion, membrane fission appears to be a slow process and is characterized by a continuous narrowing of a small pore with no final jump, while in membrane fusion, a distinct jump in pore conductance is observed prior to pore growth. The reversals in pore narrowing they have observed in the parasitic fission pore are occasionally seen in exocytotic fusion pores, but not as abruptly as seen here. Furthermore, they have seen no evidence of reopening of a completely closed parasitic fission pore (analogous to exocytotic flicker). It is tempting to regard the rapid re-enlargement of the almost-closed fission pore as a reflection of the disaggregation of, or escape from, a putative proteinaceous constricting apparatus around the cytoplasmic neck of the pore.

COMPONENTS AND KINETICS IN EXOCYTOSIS

A second group, led by **Joshua Zimmerberg,** studies molecular mechanisms involved in secretion, fertilization, and neuro-transmission, to assign functional roles to proteins and lipids involved in membrane fusion by studying the temporal dependence and properties of identifiable stages in calcium triggered cortical granule exocytosis. Their primary biochemical goal has been to learn more about the proteins which are required for membrane fusion and docking. Their primary biophysical goal has been to define the activation physiology of the calcium sensor for regulated exocytosis.

Sea urchin egg cortical granule exocytosis, an example of calcium triggered membrane fusion, has served as a model system. They have been investigating how certain proteins (NSF et al.) might regulate the exocytosis of the egg's cortical granules. One of the paradigms for NSF-dependent membrane fusion is that cytosol is able to restore activity to NEM-inactivated fusion systems. They have shown that cytosol fails to restore fusion to the NEM-inactivated isolated exocytotic machinery, even when inhibition is sub-maximal. There is no concentration of NEM whose inhibitory effect is overcome by the addition of cytosol. It has now been firmly established that cytosol has no effect upon the Ca^{2+}-sensitivity of cortical granule exocytosis in vitro. They have found that sea urchin eggs contain NSF. This protein is located in the cytosol but not in the isolated exocytotic machinery. Sea urchin egg NSF has the same membrane binding properties that have been found for NSF from other sources. In the presence of non-hydrolyzable analogues of ATP, NSF binds to the isolated exocytotic machinery. This binding is prevented by treatment with NEM and recombinant NSF competes for the same binding sites. This binding has no effect on calcium-stimulated exocytosis. These data all suggest that membrane fusion during cortical granule exocytosis is downstream of the actions of NSF. By Western blotting, they have identified proteins that cross-react with polyclonal antisera raised against the mammalian SNARE proteins, syntaxin and synaptobrevin.

The relative roles of inactivation and heterogeneity were evaluated further in the isolated planar cortex from the sea urchin eggs using multiple solution exchange protocols and perfusion at different rates. No evidence was found to support the hypothesis that either rate-dependent or rate-independent inactivation is responsible for the cessation of fusion at calcium concentrations which produced sub-maximal responses in cortical degranulation. Calcium-triggered exocytosis was heterogeneous: exocytotic responses were sub-maximal at certain calcium concentrations because all the granules capable of fusing at those concentrations of calcium had fused. Secretory models featuring calcium-dependent inactivation are inappropriate for describing cortical degranulation in the sea urchin egg. The minimum number of calcium ions involved in the fusion process was estimated by considering the behavior of a single, step-like transition at a threshold calcium concentration. This analysis suggests that more than four calcium ions are involved in triggering exocytosis. Their model, which describes both the kinetic and steady-state features observed in sea urchin cortical granule exocytosis, has been extended with the addition of a third parameter representing the activation time for fusion complexes. Discrepancies between the model and the initial time course of exocytosis have been eliminated with this modification.

The establishment of the origin of sub-maximal exocytotic responses being due to heterogeneity is important because a) differentiation between calcium-dependent inactivation and heterogeneity is required for the development of robust secretory models, b) sub-maximal responses are a feature common to all calcium-triggered exocytotic systems, and c) the goal of relating specific proteins with specific steps in exocytosis can only proceed if the steps are correctly identified. They have refined a theoretical model which can explain the complex biological behavior observed during triggered exocytosis.

THE INVOLVEMENT OF LIPIDS IN BIOLOGICAL FUSION INTERMEDIATES

The Unit on Lipid Intermediates in Fusion, led by **Leonid Chernomordik**, studies how incorporation of different lipids into fusing membranes affect different fusion stages, to further determine the fusion pathway as the sequence of the structural lipid-involving intermediates and to uncover the physical forces that drive rearrangement of two membrane lipid bilayers into one. Their working hypothesis is that the different membrane fusion systems, including fusion of purely lipid bilayers, involve a consecutive formation of two classes of transient and highly bent intermediates: stalks -- lipidic connections between contacting monolayers of fusing membranes, and pores. The stalk-pore hypothesis suggests that the energetics of hemifusion and fusion pore intermediates depends on the spontaneous curvature of different membrane monolayers.

The researchers found a lipid-sensitive stage common to fusion mediated by HA of different strains of influenza (Japan/305/57 and X-31 strains) and for hemifusion mediated by GPI-anchored HA. The effects of the different lipids correlated with their dynamic molecular shapes The lipid-sensitive stage, arrested by inverted-cone-shaped lysophosphatidylcholine (LPC) and promoted by cone-shaped oleic acid, preceded or coincided with lipid mixing as assayed by membrane dye redistribution from labeled to unlabelled cells upon their fusion. This stage was also upstream of fusion pore formation assayed both as aqueous dye transfer between cells, and by electrophysiological recording. The lipid-sensitive stage is subsequent to the low pH-induced conformational changes in HA upon its activation. The insensitivity of the LPC-arrested stage to neuraminidase, cleaving the sialic acids required for HA1- receptor binding, suggests that LPC blocks fusion at a "committed state" where the fusion peptides of HA are already inserted into the target membrane. Thus, when fusion is blocked by adding inverted-cone-shaped lipids fusion proteins still change their conformation. They can remain in this triggered conformation until the lipid block is removed by either removing added lipid or adding lipid of the opposite molecular shape. Fusion ensues, even if the triggering conditions are no longer present. Changing lipid composition is enough to release the block so that fusion goes to completion. It is as if fusion protein conformational change can do a finite amount of work, and lead to a finite reduction in the energy barrier for fusion, and lipid composition sets that energy barrier.

To clarify the fusion pathway for purely lipidic bilayers, fusion of giant unilamellar liposomes (5-15 mm in diameter) with planar lipid membranes was studied by combination of four independent assays which allowed us to distinguish a hemifusion intermediate from fusion pore formation, a fusion event from vesicle lysis, and flickering of a fusion pore from its irreversible expansion. Hemifusion was seen as membrane dye redistribution and dequenching without any release of water-soluble dye or conductance changes. Fusion pore opening was seen as a release of water-soluble dye accompanied by fast and significant increase of the measured conductance, when the high resistance of the planar bilayer was shunted by the ionic pathway via the high conductance of the permeabilized vesicle membrane, connected to the planar bilayer by the conductance of a fusion pore. The conductance measurements allowed us to distinguish fusion of vesicle to planar bilayer from vesicle lysis, and, in addition, to follow the flickering of the fusion pores. Time-resolved records of pores were surprisingly similar to pores described for viral or exocytotic fusion. This flickering fusion pore formation was often preceded by membrane hemifusion and followed by complete fusion of liposome with planar bilayer observed only for vesicles under osmotic tension. Thus, vesicle/planar bilayer fusion involves hemifusion and formation of transient fusion pores, both stages described for protein mediated biological fusion.

Although the specific protein-lipid interactions in fusion remain to be understood, the similarity between the effects of membrane lipid composition on HA-mediated fusion and those reported earlier for low pH-, Ca2+-, and GTP- dependent fusion and for the fusion of purely lipidic bilayers suggest that proteins mediate disparate fusion processes by altering elastic energies of biological membranes to facilitate bending of membrane lipids into stalk-like intermediates. The similarity of the characteristics of fusion pores, kinetics of their formation and expansion in lipid bilayer fusion with the reported characteristics of fusion pores in protein-mediated fusion allow us to draw important conclusions on the role of proteins in biological fusion. The existence of very small flickering fusion pores in exocytosis has been considered in the literature as an important evidence of the model of a proteinaceous fusion pore similar to gap junction channels. Their experiments remove this argument.

CELL INTERACTIONS IN THREE-DIMENSIONAL TISSUE CULTURE

A fourth group, led by Leonid Margolis, studies the complex cell-cell interactions in tissue which are the basis for all of the aspects of normal and pathological immune responses *in vivo*. That is particularly true for the immune system. Dysfunction of the human immune system can occur due to the impaired interactions of cells in lymphoid tissue. The most notable example of such dysfunction is HIV-disease. The ability to study the mechanisms of various immunopathological states under controlled conditions *in vitro* and within the context of human lymphoid tissue, is important for answering basic questions on immunopathogenesis including the dysregulation of the immune response caused by HIV.

HIV infection of human lymphoid tissue: a pathogenesis model. The model system (histocultures) consists of blocks of tissue maintained on a raft of collagen on culture medium. They have found that the histocultures of human tonsils they develop preserve both their cytoarchitecture, including a key element, a three-dimensional network of follicular dendritic cells, as well as an immune function. *In vitro* immunization of tonsil histocultures (but not of cells isolated from them) with tetanus toxoid (TT) results in the production of anti-TT IgG. Histocultures of lymphoid tissue are also able to support productive viral infection with two laboratory strains and six primary isolates, of syncytia-inducing (SI) and non syncytia-inducing (NSI) phenotypes, as well as macrophage- and lymphocyte-tropic viruses. Infection with HIV affects both the cellular and the functional aspects of the histocultures in an isolate-dependent manner. Infection with SI HIV-1 isolates resulted in a dramatic decline in CD4+ lymphocytes in histoculture. NSI viruses do not cause such complete depletion of tissue CD4+ lymphocytes. HIV infection greatly affected the immune response of the histocultures to tetanus toxoid. The immune response to TT was dramatically inhibited in histocultures infected with T-tropic HIV isolate. In contrast, infection with M-tropic isolate appears to lead to the enhanced production of antibodies against tetanus toxoid. Mechanisms of HIV-induced dysregulation at various stages of HIV-disease can now be studied in the context of a complex system of cell-cell interactions under controlled conditions *in vitro.*

Histocultures of human lymphoid tissue support productive HIV-1 infection with all tested primary HIV-1 isolates -1. Histocultures of human tonsils were infected with HIV-1 laboratory strains LAV.04 and SF162, and with primary infant (USA) isolates #302076, #302144, # 302074, #302056, #302143, #302151. Analysis of the media from 1308 histocultured blocks of human tonsils derived from 39 donors and infected in vitro either with one of two laboratory strains or with one of six primary isolates of HIV-1 demonstrated that all HIV isolates replicate equally well in infected histoculture. In all experiments, HIV p24 was first detected in the bathing media between days four and six after infection and its concentration rose exponentially. For all HIV isolates, a maximal p24 concentration of approximately 10^4 pg/ml was achieved by about day 10 after infection. Exponential increases of p24 concentration were also observed in tissue blocks. Virus infectious titer (TCID50/ml) in the culture medium was first detected between day one and three after infection, increasing exponentially to 10^4-10^5 on days 10 to 12 after infection. In addition, 81 histocultures of lymph nodes from 6 donors were infected with LAV.04, and each showed exponential accumulation of p24 in the media. Similar results we obtained with adenoid histocultures infected with LAV 0.4 or isolate #302144. Productive infection of human tonsils and lymph node histocultures was also confirmed by in situ hybridization detection of HIV RNA and by immunochemical detection of HIV p24.

A strong correlation exists between the extent of CD4+ T cell depletion and the isolates' SI/NSI phenotype. The average number of CD4+ cells remaining in tissue after 13 days of infection with isolates #302076 decreased to 5-15% of the control value. In contrast, after infection with isolates #302144, only 0-40% of CD4+ lymphocytes were lost from tissue, relative to controls. Regardless of whether infection led to the depletion of CD4+ lymphocytes, the average number of CD8+ cells in infected specimens was 94+7% of that in non-infected control blocks (n=11). All tested isolates fell into two distinct groups: highly and mildly depleting of CD4+ T cells. Infection with viruses of the first group, patient isolates #302076, #302143, #302151 (all SI types), resulted in a large drop in the CD4+/CD8+ ratio from day 8 to 13 compared with non-infected control tissues. After infection with LAV.04 or with #302076, the CD4+/CD8+ ratio was 20 to 25 times lower than in non-infected controls. Infection with two other isolates of the same group (#302143 or #302151) resulted in a five to eight fold decrease in this ratio. Infection with viruses of the second type, patient isolates #302144, #302074, #302056 and laboratory SF162 (all NSI-types), resulting in a CD4+/CD8+ ratio that remained at 70 to 90% of the control value. The level of depletion was not a mere kinetic consequence of infection: the CD4+/CD8+ ratio for tissue blocks infected with the NSI isolate #302144 did not decrease further when the infected histocultures were analyzed on day 20 after infection. The depletion of CD4+ lymphocytes was not dependent upon the type of anti-CD4 antibodies used in the assay. Thus the measured depletion of CD4+ lymphocytes was neither the result of masking of a particular epitope specific for the Leu 3a antibodies, nor of down-regulating the CD4 receptor in HIV-infected cells.

AZT inhibits viral replication and CD4+ T cell depletion. To test whether HIV-infected histocultures can be used to evaluate antivirals, we applied an anti-viral agent used clinically to inhibit HIV replication. Simultaneous with inoculation by primary isolate #302076, histocultures were incubated with 1 uM AZT. Such treatment significantly decreased the total viral load in the media, compared to controls. Exponential accumulation of p24 into the media bathing AZT-treated tonsil blocks was found to begin four days later than in the controls. As a result of this delay, the level of p24 in the media from AZT-treated histocultures was only one fourth to one tenth of the levels detected in the media of control histocultures at day six. Paralleling this depression of total viral load, AZT partially prevented CD4+ T cell depletion: on day six after infection, the CD4+/CD8+ ratio in the AZT-treated specimens was 50% higher than in an HIV-infected specimen without AZT. On day 11 there were almost no CD4+ T cells left in the untreated HIV-infected tissue blocks, but in AZT-treated specimens the CD4+/CD8+ ratio was 15-fold higher, almost 35% of that in the non-infected histocultures.

THE ISOLATION AND CHARACTERIZATION OF MACROMOLECULAR AND CELLULAR PARTICLES

The Section on Macromolecular Analysis, led by ***Andreas Chrambach***, pursues the improvement of analytic and preparative electrophoresis. Most of the building blocks of biological membranes and subcellular sized particles await effective separation and isolation. In relation to electrophoretic methods of separation and isolation, this may be due to the fact that in the past these separations have exploited the relatively slight differences between particles in mobility, i.e. charge/mass ratio. They are attempting therefore to introduce polymers into these separations in order to be able to separate on the basis of size and shape differences which may be more pronounced at least in some applications. They are also attempting to solubilize and separate such particles or their fragments carrying the activity of interest by application of relatively non-denaturing detergents. The isolation and characterization of cell membrane components with biological activities, and in particular that of the macromolecular complexes constituting "fusion complexes" remains a central concern of membrane biophysics at a time when effective methods for such separations and isolations have not been developed as yet.

Separation of Sea Urchin Organelles. To date, preparative electrophoretic methods for subcellular-sized particles and complexes have been in use which are responsive to net charge differences among such particles, but poorly responsive to differences in size and shape. To exploit size and shape differences among large particles, this laboratory has developed two techniques: An adaptation of preparative automated gel electrophoresis apparatus for use with concentrated polymer solutions in addition to use with gels, and a free-flow electrophoresis apparatus with capillary dimensions between the glass walls of the vessel through which the flow is conducted. During the past year, both of those techniques were successfully applied to preparative separations of subcellular-sized particles. The free-flow electrophoresis technique, using narrowly spaced glass plates, was applied to the separation of sea urchin egg vesicles. Vesicles from homogenates prepared from unfertilized and fertilized sea urchin eggs were separated by analytical CZE and preparative FFE and revealed three major fractions. These fractions were characterized by spectroscopy and DIC microscopy to reveal vesicles of different morphology. The capacity of the fractions to fuse in presence of Ca was maintained.

Separation of Liver Microsomes. The preparative mode of the automated gel apparatus was applied to rat liver microsomes. These had been show during the previous year to separate into three major electrophoretic components. Each of those was then isolated. The yield of the main component was near-quantitative after the first electroelution step, that of the two other components after no more than two steps. Each of the three fraction gave rise to identical SDS-peptide patterns, showing that their differences did not reside in the dominant elements of their protein composition. The major component exceeded the others in size, while these differed in surface net charge only. The isolated products of two of the components were not detectably impure, while a polymeric impurity in the third amounted to 10-15% by weight only. Thus, both types of preparative size separations tools have been found to be capable of yielding fractions capable of yielding functional information in future studies.

Theory of Protein Electrophoresis. Theoretical interpretation of protein electrophoresis data led to the important insight in that regard that zone dispersion, a key element of resolving power, related to interactions with either gel fibers or walls of the vessel at capillary distances. That insight guided the development of a preparative method for large particles. Availability of the band profile was to be used to numerically evaluate resolution, the central parameter of separation science, for the different electrophoretic separation methods and media providing the "molecular sieve", and to evaluate the mechanisms of band dispersion and retardation operative in those media. Previous prediction of the retardation of rigid spherical particles (proteins and charged polystyrenes) in gel electrophoresis has been based on the model of a ball penetrating a random inert network of fibers (the Ogston model). Thus, the model has been a purely geometric one, divorced from the real properties of polymer fibers. The present study aimed at correcting that situation by measuring retardation in the simplest case, that of proteins with Stokes radii of 2 - 5 nm (i.e. Mr 50 - 500 kDa), during electrophoresis in polymer solution, and attempting to account for that retardation by the predictions of the scaling theory of polymer physics as to the properties of semi-dilute polymer solutions. The retardation of proteins in polyethylene glycol (Mr 0.2 - 8 million) solutions measured in CZE scales as the 0.75th power of polymer concentration and is independent of the Mr of the polymer, as predicted by the scaling theory for semi-dilute polymer solutions. Thus, the screening length, an essential parameter descriptive of semidilute polymer solutions, is responsible for molecular sieving (size dependent retardation).

Previously, the retardation of spherical particles by gels or polymer solutions has been assumed to be a function (linear, biphasic or undulating) of particle size (radius, R) and polymer properties only. Since for a particle with $R>20$ nm that relationship was not monotonic, particle size in that range could not be derived from retardation. The present study aimed at establishing a monotonic relationship to restore this possibility. Up to R of about 20 nm, retardation and particle radius are related near-linearly. When $R < 15$ nm, this relation is independent of field strength. When $R > 20$ nm, retardation and particle size relate in a complex undulating fashion, and in dependence on field strength. However, it was found that a simple relationship exists between retardation (at a particular polymer concentration) and shear rate, defined as (mobility x field strength)/R.

Peak asymmetry provides information concerning the mechanism of peak spreading and resolution as a function of time and polymer concentration (the "Giddings-Weiss model", Weiss et al., 1996).

Peak asymmetry was measured in CZE in polymer solution over a wide particle size range (R = 3 - 100 nm) with the aim of elucidating the sieving mechanism in polymer solution in the manner previously applied to gels. Band width and asymmetry are proportional. Both increase with polymer (polyacrylamide, 5 million Mr) concentration in the size range of proteins (R < 7 nm) independently of field strength, but vary in concave relation with polymer concentration and in dependence on field strength for larger particles (up to R = 100 nm). The asymmetry behavior in the smaller protein size range can be accounted for by the Giddings-Weiss model; that in the larger size range remains unaccounted for mechanistically.

It is widely assumed that bands in gel electrophoresis spread by diffusion with increasing migration time. However, previous studies on DNA (Yarmola, E. and Chrambach, A., 1995) and phycoerythrin (Chen, N., unpublished data) had shown that macromolecules don't diffuse appreciably in gels in absence of a field. Moreover, the deviation from Gaussian peak shape of phycoerythrin and conalbumin had suggested that the entanglement of the particle with the gel fiber, not diffusion, is predominantly responsible for band asymmetry, and therefore band spreading, in gels (Weiss et al., 1996). Therefore, the aim of the present study was to solidly establish that diffusion was not a significant element of macromolecular band spreading in gels. DNA of 1, 2 and 3 kb length were subjected to gel electrophoresis with measurement of band spreading. The power supply was turned off, and band spreading in the absence of an electric field was measured. Expressing the rate of band spreading as an electrophoretic dispersion coefficient and a diffusion coefficient in absence of a field, respectively, the ratio of the two was found to be 5/1, 15/1 and 45/1 for 1, 2 and 3 kb DNA respectively.

To construct a model predicting band spreading in gels, a systematic study of the spreading of phycoerythrin bands as a function of time, load, gel concentration, ionic strength and field strength was carried out. Phycoerythrin band variance varies non-linearly (close to parabolically) with time of electrophoretic dispersion and linearly with time of diffusion in the absence of a field. Peak area decreases slightly in proportion to migration time. Since the area is proportional to load, the decline of area with migration time increases with increasing protein load. Variance of electrophoretic dispersion increases as the field strength is lowered, while band spreading is independent of field strength during diffusion. Log (peak amplitude) decreases with log (migration time) linearly. Resolution increases with migration time to a constant plateau value. A mathematical model consistent with these data was devised which allows one to predict dispersion in gel electrophoresis.

Improved Resolution of the Automated Electrophoresis Apparatus. The Joule heat dissipation capacity of gel electrophoresis apparatus is usually poor. That of automated apparatus extends to about 20 Watts, limiting the field strength applicable to that apparatus to approximately 15 V/cm with gels of 3 mm thickness, or 45 V/cm with those of 0.5 mm thickness (Chang, H.-T. and Chrambach, A., 1996). The value was increased to 80 V/cm by use of isoelectric histidine as the gel buffer (Chen, N. and Chrambach, A., 1996). Since resolution increases with field strength, the present study aimed at further increasing it, using a pulsed power supply to provide periodic cooling of the gel. Resolution between two representative proteins at 80 V/cm is doubled when 10 s of electrophoresis are periodically interspersed by 120 s in the absence of an electric field. The field strength could not be increased beyond that value since the limit of power supply is 1,000 V in the HPGE-1000 apparatus; but the doubling of resolution suggests that with a 2-3,000 V power supply (that would require rewiring of the instrument) field strengths in the hundreds of V/cm could be achieved, similar to CZE, and with the same effect of raising the resolving capacity.

A major handicap to the wide acceptance of automated gel electrophoresis apparatus with intermittent scanning of fluorescence has been the requirement for fluorescent labeling of non-fluorescing compounds. Such labeling not only is likely to modify the conformation of the molecule to an unknown degree, it also decreases the net surface charge and therefore the mobility in direct relation to the degree of labeling. A previous method of fluorescence detection of unlabeled compounds used an uncharged fluorescing agent (umbelliferone) in the gel, but appeared undesirable in view of the possibility of chemical interactions with that agent. Interposition of fluorescent yellow paper between the electrophoresis cell and the underlying mirror of the HPGE-1000 apparatus raises the fluorescent background of the gel scan to the top of the scan recording. Since the range of excitation wavelengths (280-360 nm) coincides with the absorbance peak of proteins at 280 nm, the intensity of the incident excitatory light is reduced by the absorbance of a protein, and the resulting fluorescence reduction produces an inverted band by which the unlabeled protein is detected. At low protein concentrations, the sensitivity of protein detection by fluorescence reduction is reduced by only a factor of 2 compared with that of fluorescence detection. At high protein concentrations, the sensitivity relative to fluorescence is reduced by a factor of up to 20. The detection sensitivity of the fluorescence reduction can be increased by impregnating the yellow paper with the fluorescence enhancer, Na-salicylate.

In a representative application of automated gel electrophoresis to proteins, elimination of a mirror below the gel tray increased the sensitivity of fluorescence detection (peak amplitude/average noise amplitude) by a factor of 6.3. That increase in detection sensitivity allows for lowered sample loads by which the band width is reduced and resolution is increased.

Automated Recovery of Proteins. Although intermittent scanning during separation is automated, the isolation has not been: After separation, bands were manually detected by UV-lamp and manually withdrawn by syringe. The present project aimed at restoring automation to that procedure. Automation was restored to the isolation procedure from concentrated polymer solutions by 3 instrumental modifications on the HPGE-1000 apparatus: i) A sample cup insert holding a thin Teflon tube in the cup was constructed and the arms holding the sample cup in the apparatus were elongated so that the bottom edge of the cup reached 2 to 2.5 mm into the polymer solution of 3 mm height; ii) the syringe by which the band of interest is withdrawn was connected to the insert by a thin Teflon tube of sufficient length to allow the sample cup any position along the migration path; iii) a hole was drilled into the cover of the HPGE-1000 apparatus to allow for the tube connection between syringe and sample cup; the hole was covered by mounting the syringe solidly in a frame on top of the instrument cover. These instrumental modifications allowed one to position the sample cup over the band of interest automatically and to withdraw the band by syringe while the sample cup was automatically placed into the desired position.

Derivitized Agarose Yields Improved Resolution of Proteins over Polyacrylamide. It is widely assumed that polyacrylamide gels excel in their resolving capacity for proteins over agarose gels. Also, the commercial literature suggests that some agaroses such as MetaPhor-XR exhibit a greater resolving capacity for proteins than other agarose types. The possibility to quantitate resolution by use of scanning apparatus as a function of migration time and gel concentration opened up a way to test those two assumptions. Measurements of migration distances and band widths on two representative proteins show that SeaPrep agarose, 10% derivatized with hydroxyethyl residues and therefore highly soluble, exceeds polyacrylamide in resolving power for proteins by a factor of 4-5. MetaPhor-XR, Gold, SeaKem GTG have less resolving capacity than SeaPrep. The most widely used polyacrylamide gels are those used for analysis of SDS-polypeptides in a single dimension or in the second dimension. The effectiveness of SeaPrep agarose gels or pore gradient gels made from it will be tested in those applications. Substitution of polyacrylamide by agarose should result in improved reproducibility of mobilities, since the former is the product of a complex free radical polymerization reaction, the latter a product of simple thermally controlled gelation.

MASS SPECTROMETRY

The Section on Mass Spectrometry and Metabolism, led by ***Alfred Yergey,*** has continued to apply the knowledge of the physical chemistry of ions in their gas phase to basic biomedical research. In their project on the dynamics of the growth and development of bone, they strive to determine the dynamics by which calcium is accreted to and removed from bone during human growth and development. An overall understanding of the developmental aspects of calcium absorption, excretion and distribution to bone as a function of development in humans is emerging as a consequence of these studies. Their results can begin to be used to interpret abnormalities of calcium metabolism in a manner that can be therapeutically useful. To date this objective has been met by studies of absorption and distribution of calcium in healthy infants and children, in children with diseases suspected of altering calcium metabolism and in healthy women in altered states of calcium metabolism such as pregnancy and lactation. The objective of the thermodynamic investigations is to determine solvation enthalpies of ions of interest using gas phase ion molecule chemistry under conditions of either equilibrium or low pressure collisional dissociation. Under these circumstances, ions, the solute species of interest, can be viewed as isolated entities surrounded by selected numbers of water molecules. The objective of the conformational studies is determination of solvent and structural dependent alterations in peptide conformations as measured by the rates at which labile hydrogens are exchanged in solutions of heavy water. In the long term they see these objectives leading to an increased understanding of the fundamental aspects of protein and peptide folding and the basic thermodynamics that underlie the ordering of membranes.

Calcium Metabolism. The principal methodology used employs stable isotopes of calcium as tracers for the entrance and distribution of calcium in the body. Determination of isotope balances allows the calculation of important parameters of calcium metabolism, principally the rates of absorption and excretion. The effect of recombinant human growth hormone, hGH, treatment on Ca metabolism and growth is continuing to be investigated in patients with Osteogenesis Imperfecta Type III in collaboration with J. Marini, HDB. These patients are studied before and after treatment with hGH. Preliminary results of calcium metabolism/distribution studies in 5 subjects studied pre and post treatment show an apparent decrease in fractional absorption of dietary calcium (0.29±0.19 pre treatment vs. 0.14±0.11 post treatment). At the same time an increase in both the whole body exchangeable pool (TEP = 265±95 mg/kg pre treatment vs.

673±616 mg/kg post treatment) and rate of calcium accretion to bone (Vo+ = 94±29 mg/kg/d pretreatment vs. 142±75 mg/kg/d post treatment) was observed. While suggestive of possibly important consequences, the variance in the mean values of these results is too great to be significant at the present time. They are conducting further studies to confirm these observations and are in the process of analyzing 6 additional studies undertaken in the past year.

Small water cluster measurements. Measurement of the enthalpy change for the successive additions of water molecules to a hydrated proton cluster ion are the archetypal reaction for studies of hydration. This reaction has been studied extensively in the past, and is being used as the model and proof of method in the present work. Since the hydrophobic quaternary ions initially of interest can be introduced to the ion source from a flowing water stream, use of the water cluster system as the starting point for measurements is a reasonable approach. The investigators have obtained mass spectra of water clusters of the proton in the equilibrium source for which the electric fields and temperatures within the source are consistent with equilibrium. Ion intensity ratios consistent with equilibrium for well determined concentrations of water vapor will be available in the near future. Thus these ion/water systems are reasonable models for assessing the nature of the structure of water in the vicinity of an ion. They have the ability to measure the energetics of these associations. Such models and measurements are fundamentally important to an understanding of basic biological processes.

Large water cluster measurements. CID studies of water clusters in the region of the magic number clathrate have been made. Determination of the energy minima for the processes awaits some refinement in data analysis techniques, but preliminary values obtained for the processes are consistent with published values for solvation enthalpies of the proton. The behavior of a number of organic and inorganic ionic species has been examined for their ability to form magic number clathrates with water under electrospray ionization conditions. To date, the only hydrated ions which have been reported to form the stable clathrate cages are hydronium and ammonium, H_3O^+ and NH_4^+. They have found that some of the tetraalkyl ammonium ions, R_4N^+, where R varies from methyl through pentyl as well as some of the alkali and alkali earth metal ions form such clathrates. Specifically, the tetraethyl through tetrapentyl ammonium ions, K^+ and Cs^+ all give rise to the 20 member anomaly in the water cluster distribution, while the tetramethyl ammonium, Li^+, Na^+, Mg^{2+}, Ca^{2+} and Ba^{2+} ions do not. Current theories of water structure in the vicinity of ions may serve to account for these observations.

Solvent water is considered to be organized around solute ions by either electrostatic or hydrogen binding forces, or some combination of these. One might expect that ions with high surface charge densities to organize water principally on the basis of electrostatic forces, due either to small radii or large numbers of charges. On the other hand, ions with low charge densities might be expected to organize water by hydrogen binding forces. At first, one might think that the alkali and alkali earth ions would organize water electrostatically while the tetraalkyl ammonium ions would organize it through hydrogen bonding. Evidence from solution thermodynamics shows, however that there are transitions in solvation enthalpies that occur between Na^+ and Cs^+ and similarly between tetramethyl and tetrapropyl ammonium ions with the behavior of the K^+ and tetraethyl ammonium ions being somewhat intermediate. These transitions are ascribed to changes in the nature of water's organization around the ions. The present results may represent direct observation of the such transition behavior, and as such, are confirmatory of some of the current theories of the nature of bulk water structure.

Angiotensinogen II. Studies of protein/deuteron exchange of angiotensin II under solvent flow conditions using electrospray ionization have been used as a model for studies of determining peptide conformation. There are 16 protons on the angiotensin II molecule that may be considered labile enough to exchange with solvent protons. Rates of exchange are expected to be fast for protons that are exposed to the solvent and slower for those that are protected by reason of conformational folding. 12 of the 16 labile protons exchange with the solvent within an average of 0.02 minutes while the remaining four exchange on the average within 4.0 min. Controlled fragmentation of the angiotensin II molecule in the ion source following the proton exchange chemistry has allowed the slowly exchanging protons to be identified. Two of them are the amide protons bracketing the proline in the center of the molecule and give rise to a ß-turn in its center. The remaining two are associated with each terminal residue and give rise to a second planar region in the molecule.

In order to investigate the possibility of conformational changes that would be associated with interactions with a lipid, the angiotensin II was mixed with cholic acid, which forms lipid micelles of small aggregation number at low concentration. Proton exchange was investigated by rapidly diluting the peptide/micelle mixture in D_2O and introducing the diluted material directly into the mass spectrometer. While any peptide/micelle complex was destroyed by the ionization process, this study showed a very different exchange pattern than had been seen in the earlier one. Three labile hydrogens, associated with the lipophyllic side chains of the central portion of angiotensin II did not exchange with the solvent in the course of 4 hr. This is consistent with that portion of the peptide being bound or dissolved in a lipid micelle. These studies of H/D exchange are the first to report exchange rates for each labile hydrogen using mass spectrometry. In addition, there are no published data that report alterations of a peptide conformation for a chemical interaction other than changing solvent. This study is a potential model system for describing detailed conformational changes of peptide membrane interactions.

ß-amyloid protein. The mass spectra of ß-amyloid protein (1-40) associated with neuritic placques of Alzheimer's Disease have unambiguously shown a conformational change in the peptide that is related to neurotoxicity. Electrospray mass spectra of fresh samples of synthetic peptide, ßA4, at concentrations ranging from 250 μM to 6μM have shown the presence of a mixture of monomeric and aggregated forms of the peptide. The aggregates range in size from dimer to tetramer and are more abundant at the higher concentrations. The most abundant ion of the monomeric form arises from the addition of 5 protons to the molecule, while the most abundant dimer results from the addition of 9 protons. As the original fresh samples are aged by their containers being placed in water baths at 37°C, they observe a decrease of about 0.6 for the average charges associated with the monomer. This is interpreted as a loss in charge site availability and is associated with a conformational change in the monomer to a more compact form. At the same time, the aged samples show no loss in average charges associated with the aggregate forms, but exhibit a loss in their intensity relative to the monomers. They interpret these observations as being the consequence of a relatively "open" conformation of the ßA4 being present in fresh solution. This open form is capable of forming aggregates in a dynamic equilibrium which leads to reduced aggregation upon dilution. Upon aging, the "open" monomeric form alters to a more compact form with a loss in average charge and a reduction in the abundance of aggregates of the "open" form.

This interpretation is consistent with published circular dichroism evidence which shows a random coil form of ßA4 shifting to a ß-sheet form with aging. The results of the mass spectrometry of ßA4 peptide are the first to show any differences between toxic and non-toxic forms of the peptide. That is potentially significant for fundamental studies in vitro of this material that are directed to investigations of the basis for the conformational change. These results are also significant because they show the subtlety of the structural effects that can be probed by electrospray ionization mass spectrometry.

NIH/NASA CENTER FOR TISSUE CULTURE

A new center for tissue culture and tissue microscopy is now available to intramural scientists. This center, funded jointly by NICHD and NASA, features a novel rotating wall vessel (NASA Bioreactor) for maintaining and promoting both the original differentiated state of various tissues and the association and differentiation of isolated cells into tissue-like aggregates. To evaluate tissue function, the Center is providing a facility for imaging which includes image processing and single confocal and dual photon scanning laser microscopy. NIH scientists are invited to submit a short proposal for pilot projects. Bioreactors, supplies, and trained technical support are available to investigate the fate of cells or tissues cultured from one cell type or cocultures, thin tissue slice culture, or suspended aggregate or tissue culture. Histology and confocal microscopy are provided to evaluate results. Principal investigators must provide direction concerning specific aspects of their project. Principal investigators who are successful in obtaining preliminary data in phase 1 indicating utility of the Bioreactor for solving important problems are invited to submit a proposal for funding of a limited number of projects. The following is a list of ongoing projects.

Differentiation of salivary cell culture in the RWV bioreactor PIs: Hynda Kleinman and M. Hoffmann. RWV is used to investigate the differentiation of salivary cells interacting with extracellular matrix in the anisotropic conditions of RWV suspension. When HSG cells are cultured on an extract of basement membrane (which is an extracellular matrix that normally contacts acinar and ductal cells in vivo), they undergo acinar differentiation and thus are a model to study salivary gland acinar. RWV provides the opportunity to study this phenomena in cells not attached to solid substrate.

Protein crystal growth in Bioreactor. PI Dr. Adrian Parsegian, Laboratory of Structural Biology, Division of Computer Research and Technology, NIH

The bioreactor may provide clues as to why protein crystals stop formation at certain sizes and to better understand the forces that affect protein crystal growth and structure. Crystals formed in the Bioreactor will be compared to those formed through conventional methods to evaluate whether there are differences in size, structure, and symmetry of the crystals.

Development of long-term three dimensional cultures of intestinal tissue culture cells in the RWV bioreactor to study the interaction of bacteria and parasites with these differentiated cells. PIs.: Darcy E. Hanes, Marianne Miliotis and Ben D.Tall, Food and Drug Administration, Washington, DC.

The first goal of this project is to obtain a tissue culture model that mimics *in vivo* intestinal tissue.. The interaction of enteric pathogens with these cells will be examined to determine the mechanisms used by the bacteria to cause disease. Differentiated intestinal cells are also required to maintain certain intestinal parasites.

Culture of sections of neonatal rat hypothalamus in the Bioreactor in an effort to maintain the general cytoarchitecture and functionality of the tissue, in particular, the neurosecretory pathways. PI. Harold Gainer, Division of Intramural Research, National Institute of Neurological Disorders and Stroke.

Thin sections of rat hypothalamus will be cultured in the Bioreactor and then analyzed to determine if structure and function have been maintained. The Bioreactor should allow the tissue to be cultured without significant loss of cells, overgrowth of certain cell populations, or loss of neurosecretory function. The tissue will be examined histologically for maintainence of general structure including the general hallmarks associated with each particular slice of the hypothalamus and they will be stained for the presence of oxytocin and vasopressin secreting cells.

Human lymphoid tissue in the Rotating Wall Vessel: A two-compartment model of the lymphoid system for studying HIV infection in vitro. PI Leonid Margolis and Joshua Zimmerberg, LTPB, National Institute of Child Health and Development, NIH.

The goals are: (i) to facilitate the survival and preservation of the native cytoarchitecture of explanted blocks of human lymphoid tissue cultured in the RWV Bioreactor, (ii) to develop methods for re-supplying new lymphocytes to the RWV cultured human lymphoid tissue, (iii) to study the contribution of recirculatory traffic of HIV-infected peripheral lymphocytes to the pathogenesis of HIV infection in human lymphoid tissue

Differentiation in human spleen, lymph node, and bone marrow : A three-compartment RWV based model for studying T-cell differentiation and lymphocyte traffic. PI Steven Hatfill, National Institute of Child Health and Development, NIH.

The goal of the project is to facilitate the survival and preservation of the native three-dimensional cytoarchitecture of explanted biopsies of human bone marrow and its functionality with respect to the production of early T-cell precursors in a fluidized bed bioreactor, (ii) To facilitate the survival and preservation of the native three-dimensional cytoarchitecture of human tissue explants of the spleen, tonsil and lymph node in the RWV Bioreactor during co-culture, (iii) To study the traffic of T-cell precursors from the cultured bone marrow to RWV co-cultured explants of human spleen and adult thymus and to determine if double positive CD4+/CD8+

precursor cells can mature into separate CD4+ and CD8+ lymphocyte subpopulations within the spleen.

RWV Bioreactor models of human squamous metaplasia. PIs: Steven Hatfill, Paul Duray, Department of Pathology, National Cancer Institute, NIH.

Metaplasia, the transformation of one adult cell type within a tissue into another cell type is thought to be a precursor to neoplasia RWV bioreactor is utilized for genetic studies concerning the metaplastic phenotype of human epithelial cells within their native tissue microenviroment. The major result the RWV Bioreactor was able to maintain a pre-existing metaplastic epithelial phenotype. For the first time, there is now a system for studying the pathogenesis of metaplasia and may allow the study of its progression to neoplasia.

Long term maintenance of human prostate tissue in the RWV Bioreactor. PIs: Steven Hatfill, LTPB, National Institute of Child Health and Development, and Paul Duray, Department of Pathology, National Cancer Institute, NIH.

The primary focus of these experiments is to develop a high-fidelity in vitro model of normal prostate tissue structure and function which is suitable for testing the hypothesis that certain pesticide residues may cause a disruption of normal prostatic function and that a loss of heterozygosity at the 8p12-21 locus is responsible for the invasive nature of primary prostatic cell carcinoma. RWV Bioreactor is utilized develop a long duration in vitro tissue model of the human prostate suitable for the investigation of prostatic epithelial pathobiology.

Studying of differentiation of human dendritic cells PI. Peter Cohen DCT. NCI, and Charles. Carter Dept. of Transfusion Medicine, CC, NIH

Differentiation of dendritic cells involves interactions of many lymphoid cells with each other. The RWV Bioreactor provides the way to study the differentiation of dendritic cells from monocytes by modifying the microenvironment of the precursor cells.

Evaluating the role of gap junctions in lymphoid tissue in RWV Bioreactor. PI. Andrew Harris , NASA/NIH Center

The role of gap junctions in the structure and function of human lymphoid tissue cultured in RWV will be evaluated. Various stimulators and inhibitors of gap junction channels will added to the media and their effect on the tissue will be assessed. Cells of lymphoid tissue will be reaggregated and gap junctions will be evaluated in theses aggregate in order to correlate with the structure and function of reaggregated tissue.

RWV Bioreactor models of three-dimensional host-tumor interactions in human carcinoma. PIs: Paul Duray, Dept. Of Pathology, National Cancer Institute and Steve Hatfill, LTPB, National Institute of Child Health and Development, NIH.

RWV Bioreactor is used as a culture system to study the microenviroment in which host extracellular matrix and tissue cells interact with infiltrating metastatic tumor cells.

In Vitro production of Herpes Zoster Virus using human skin explants in the RWV Bioreactor. PIs Jeffery Cohen, Department of Virology, National Institute for Allergy and Infectious Diseases, and Steven Hatfill, LTPB, National Institute for Child Health and Development.

The goal is explore the types of tissue and host/virus or host/bacterial relations that can be successfully maintained in the RWV culture system, we will attempt the productive infection of RWV cultured human skin explants with the Herpes Zoster virus. This virus was chosen because of the difficulty in producing cell free virus in previous tissue culture systems, and the complexity of the epithelial layer which characterizes human skin.

In Vitro Hepatocyte infection by avian malaria in the RWV Bioreactor. PIs Helmut Ziegler, National Institute for Allergy and Infectious Diseases, Laboratory of Parasitic Diseases, NIH, and Steve Hatfill, LTPB, National Institute for Child Health and Development, NIH.

The goal of the project is to culture the cryptozoite stage of avian Plasmodium sp. within the context of a normal three dimensional hepatic tissue microenviroment.

RWV Bioreactor model of lung adenocarcinoma invasion and metastasis .PI:. Paul H. Duray, Dept. of Pathology, NCI,

To develop a bioreactor model of lung adenocarcinoma suitable for assessing the effectiveness of various chemotherapeutic agent combinations in vitro. Patient lung adenocarcinoma biopsy material will be introduced into the system and both the local invasion and the metastasis into non-pulmonary tissue assessed.

LABORATORY OF THEORETICAL AND PHYSICAL BIOLOGY - 1996

Chief, Joshua Zimmerberg, M.D., Ph.D.

Principal Investigators
Andreas C. Chrambach, Ph.D.
Alfred L. Yergey, Ph.D.

Tenure-Track Investigators
Leonid Chernomordik, Ph.D.
Leonid Margolis, Ph.D.

Senior Fellows
Svetlana Glushakova, Ph.D.
S. Vogel, Ph.D.

Post-Doctoral Fellows
Paul Blank, Ph.D.
Huan-Tsung Chang, Ph.D.
Alexandr Chanturiya, Ph.D.
Nong Chen, Ph.D.
Steven Hatfill, M.D., Ph.D.
David Kingsley, Ph.D.
Evgenia A. Leikina, Ph.D.
Ilya Plonsky, Ph.D.
Sergey Radko, Ph.D.
Alexander Sokoloff, Ph.D.
Masahiro Tahara, M.D., Ph.D.
Kim Timmers, Ph.D.

Pre-Doctoral Fellow
Stacy Tutt

Guest Researchers
Boris Baibakov, Ph.D.
Steven Hollenberg, M.D., Ph.D.
Kimberly O'Brien, Ph.D.
Helena Pulyaeva, Ph.D.
Vladimir Ratinov, Ph.D.

Senior Technical Specialist
Nancy Vieira

LTPB - FY96 Bibliography

Chang HT, Chrambach A. Application of gels of 0.5 mm thickness to electrophoresis in the automated HPGE-1000 apparatus: Improved resolution. Electrophoresis, 1996;17:80-83.

Chang HT, Zakharov SF, Chrambach, A. Separation and isolation of subcellular-sized particles by electrophoresis in polymer solution, using the commercial scanning apparatus. Electrophoresis, 1996;17:776-780.

Chanturiya AN, Nikolaenko AN, Shatursky OYo, Lishko VK. Probing the structure-function relationship of α-latrotoxin formed channels with antibodies and pronase. Toxicon, 1996, in press.

Chanturiya AN. Filter switching device for dual wavelength videoimaging. J Fluorescence, 1996, in press.

Chanturiya AN. A comparative study of planar lipid membranes formed by Montal-Mueller and Mueller-Rudin techniques. Membr Cell Biol, 1996;13:235-240.

Chen N, Chrambach A. Application of the commercial gel electrophoresis apparatus with intermittent fluorescence scanning to a non-fluorescing protein. Anal Biochem, 1996, in press.

Chen N, Chrambach A. Enhanced field strength and resolution in gel electrophoresis upon substitution of buffer by histidine at its pI. Electrophoresis, 1996;17:699-703.

Chen N, Chrambach A. Improved resolution in the gel electrophoresis of proteins by a periodically interrupted electric field. J Biochem Biophys Methods, 1996, in press.

Chernomordik LV. Non-bilayer lipids and biological fusion intermediates. Chem and Physics of Lipids, 1996, in press.

Chrambach, A. Quantitative and automated electrophoresis in sieving media: Past, present and future. Electrophoresis, 1996;17:454-464.

Galkina SI, Sud'ina GF, Margolis LB. Regulation of intracellular ph by phospholipase a2 and protein kinase c upon neutrophil adhesion to solid substrata, FEBS Letters 1996, in press.

Goans RE, Weiss GH, Vieira NE, Sidbury JB, Abrams SA, Yergey AL. Calcium Kinetics in Glycogen Storage Disease. Calcif Tiss Int, 1996, in press.

Kalkwarf HJ, Specker BL, Heubi JE, Vieira NE, Yergey AL. Intestinal Calcium Absorption of Women during Lactation and after Weaning. Am J Clin Nutr, 1996;63:526-531.

Kaplan DJ, Bungay P, Sullivan J, Zimmerberg J. A rapid-flow perfusion chamber for high-resolution microscopy. J Microscopy, 1996;181:286-297.

Radko SP, Chrambach A. Parallelism between width and asymmetry of peaks of rigid, spherical particles in CZE using polymer solutions. Appl Theor Electrophoresis, 1996, in press.

Radko SP, Chrambach A. Electrophoresis in polymer solutions: Mechanisms of "molecular sieving". J Phys Chem, 1996, in press.

Radko SP, Chrambach A. Mechanisms of retardation of rigid spherical particles with 3 to 1,085 nm radius in capillary electrophoresis, using buffered polyacrylamide (Mr=5 million) solutions. Electrophoresis, 1996, in press.

Sokoloff AV, Whalley T, Zimmerberg J. The characterization of N-ethylmaleimide-sensitive thiol groups required for the GTP-dependent fusion of endoplasmic reticulum membranes. Biochem J, 1995;312:23-30.

Suss-Toby E, Zimmerberg J, Ward G. Toxoplasma invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore. Proc Nat'l Acad Sci USA, 1996;93:8413-8418.

Vogel SS, Blank PS, Zimmerberg J. Poisson distributed active fusion complexes underlie the control of the rate and extent of exocytosis by calcium. J Cell Biol, 1996, in press.

Weiss GH, Sokoloff H, Zakharov S F, Chrambach A. Interpretation of electrophoretic band shapes by a partition chromatographic model. Electrophoresis, 1996, in press.

Xu Y, Zhang X, Yergey AL. Studies of Transition Metal Ion Complexes with Amino Acids and Peptides by Electrospray Ionization Mass Spectrometry. J Am Soc Mass Spectrom, 1996;7:25-29.

Yarmola E, Sokoloff H, Chrambach A. The relative contributions of dispersion and diffusion to band spreading (resolution) in gel electrophoresis as a function of DNA length. Electrophoresis, 1996, in press.

Yergey AL. Stable Isotopic Tracers in Mineral Metabolism. J Nutr, 1996;126:355S-359S.

Zakharov SF, Chang HT, Chrambach, A. Reproducibility of mobility in gel electrophoresis. Electrophoresis, 1996;17:84-90.

Project Numbers - Annual Report 1996

LABORATORY OF THEORETICAL AND PHYSICAL BIOLOGY (LTPB)
[Name changed to Laboratory of Cellular & Molecular Biophysics (LCMB)]

ZO1 HD 00165-21	Isolation and Characterization of Macromolecular and Cellular Particles Andreas C. Chrambach, Ph.D.
ZO1 HD 00171-20	Electrophoretic Methodology Andreas C. Chrambach, Ph.D.
ZO1 HD 01400-14	Dynamics of the Growth and Development of Bone Alfred L. Yergey, Ph.D.
Z01 HD 01409-11	Membrane Remodeling during Viral-Induced Syncytia Formation and Parasitic Invasion of Cells Joshua Zimmerberg, M.D., Ph.D.
Z01 HD 01415-06	Components and Kinetics in Exocytosis Joshua Zimmerberg, M.D., Ph.D.
Z01 HD 01416-04	Cell Interactions in Three-Dimensional Tissue Culture Leonid Margolis, Ph.D.
ZO1 HD 01417-03	Energetics of the Interaction Between Water, Membranes, and Macromolecules Alfred L. Yergey, Ph.D.
ZO1 HD 01418-02 **TERMINATED**	Regulation of Mitosis at the Cellular Level Frank A. Suprynowicz, Ph.D.
ZO1 HD 01419-02 **TERMINATED**	Localization of Membrane Fusion Proteins Joshua Zimmerberg, M.D., Ph.D.
Z01 HD 01420-01 **TERMINATED**	Calcium Triggered Membrane Trafficking in Sea Urchin Eggs Steven Vogel, Ph.D.
ZO1 HD 01501-05	The Involvement of Lipids in Biological Fusion Intermediates Leonid V. Chernomordik, Ph.D.

ZO1 HD 01421-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00165-21 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation and Characterization of Macromolecular and Cellular Particles

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Chrambach	Head	SMA, LTPB, NICHD
Others:	S. Vogel	Visiting Associate	SMCB, LTPB, NICHD
	S. Radko	Visiting Fellow	SMA, LTPB, NICHD
	N. Chen	Visiting Fellow	SMA, LTPB, NICHD

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Macromolecular Analysis

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To date, preparative electrophoretic methods for subcellular-sized particles and complexes have been in use which are responsive to net charge differences among such particles, but poorly responsive to differences in size and shape. To exploit size and shape differences among large particles, this laboratory has developed two techniques: An adaptation of preparative automated gel electrophoresis apparatus for use with concentrated polymer solutions in addition to use with gels, and a free-flow electrophoresis apparatus with capillary dimensions between the glass walls of the vessel through which the flow is conducted. During the past year, both of those techniques were successfully applied to preparative separations of subcellular-sized particles. The free-flow electrophoresis technique, using narrowly spaced glass plates, was applied to the separation of sea urchin egg vesicles. Vesicles derived from fused and non-fused sea urchin eggs, and, more importantly, fertilized and unfertilized eggs, were separated. The capacity of the fractions to fuse in presence of Ca was maintained. The preparative mode of the automated gel apparatus was applied to rat liver microsomes. These had been shown during the previous year to separate into three major electrophoretic components. Each of those was now isolated. The yield of the main component was near-quantitative after the first electroelution step, that of the two other components after no more than two steps. Each of the three fractions gave rise to identical SDS-peptide patterns, showing that their differences did not reside in the dominant elements of their protein composition. The major component exceeded the others in size, while these differed in surface net charge only. The isolated products of two of the components were not detectably impure, while a polymeric impurity in the third amounted to 10-15% by weight only. Thus, both types of preparative size separations tools have been found to be capable of yielding fractions capable of providing functional information in future studies.

Project Description:

The project aims at applying advances in separation technology reported in the joint Project Report to macromolecular and cellular particle separations of biological importance. In particular, that technology comprises quantitative and automated modes of electrophoresis in gels and polymer solutions, and associated instrumentation and software.

Objective:

Most of the building blocks of biological membranes and subcellular sized particles await effective separation and isolation. In relation to electrophoretic methods of separation and isolation, this may be due to the fact that in the past these separations have exploited the relatively slight differences between particles in mobility, i.e. charge/mass ratio. We are attempting therefore to introduce polymers into these separations in order to be able to separate on the basis of size and shape differences which may be more pronounced at least in some applications. We are also attempting to solubilize and separate such particles or their fragments carrying the activity of interest by application of relatively non-denaturing detergents. The isolation and characterization of cell membrane components with biological activities, and in particular that of the macromolecular complexes constituting "fusion complexes" remains a central concern of membrane biophysics at a time when effective methods for such separations and isolations have not been developed as yet.

This year's objective was the isolation and subsequent characterization of subcellular-sized particles such as electrophoretic microsomal components and sea urchin egg granules. The past year had produced evidence of a number of such components in capillary zone electrophoresis, using polyacrylamide (of molecular weight 5 million) solutions as the "molecular sieving" medium. Since the pattern of peaks was too complex to allow for the construction of Ferguson plots of these components (in view of the fact that mutual overtaking of bands upon variation of polymer concentration could not be ruled out), isolation became necessary for Ferguson plot analysis. Capillary zone electrophoresis does not lend itself to isolation in the required amounts. Thus two approaches were attempted which promised to yield the required isolation method - automated apparatus using agarose gels and solutions, and a modified Hannig apparatus in conjunction with polymer solutions.

Subproject (i): Capillary electrophoresis separation and free-flow electrophoretic isolation of sea urchin egg granules (Radko, S. P., Tchestkov, V., Vogel, S. S. and Chrambach, A.)

Objective:

Our goal is to apply recent developments in electrophoretic separations of large macromolecular assemblies to isolate and characterize the intracellular vesicles of sea urchin eggs before and at various times after fertilization. A comparative study of the proteins which reside on sea urchin egg vesicles will further our knowledge of the mechanism of exocytotic granule transport, docking, membrane fusion, and endocytotic membrane retrieval. In turn, this knowledge may be applied to various aspects of medicinal chemistry including drug screening and drug delivery. For such an investigation, the cortical granules associated with the sea urchin egg are an abundant source. They are biologically significant since they exhibit fusion activity either by themselves or in association with the membrane, in response to changes in Ca concentration. After fertilization, other exocytotic granules migrate from the cytoplasm to the cell surface, dock and fuse over the next seven days of development. Other activities include compensatory endocytotic activity to maintain egg surface area and volume after fusion of cortical granules. The initial objective of the study was to find conditions of granule separation, using analytical CZE and preparative FFE.

Methods Employed:

Sea urchin eggs were homogenized in a low ionic strength buffer (1M glycine, 5 mM PIPES, pH 6.7, 5 mM EGTA, and 500 M BAPTA). Vesicles in the homogenate were separated by free-flow electrophoresis (FFE) on an Octopus apparatus (Dr. Weber GmbH, Munich, Germany) and by CZE in a Beckman P/ACE 2100 apparatus. FFE fractions are collected and characterized by spectroscopy, microscopy, and SDS-page electrophoresis. Fluorescent endosomes were formed and isolated as follows. Eggs were incubated at room temperature in 100 m rhodamine dextran (3,000 MW) and activated with sperm or calcium ionophore. After 15 minutes the reaction is stopped by placing the reaction on ice and subsequent centrifugation is used to remove fluorescent dextran from the bulk-solution. Eggs are then processed as described above.

Significance to Biomedical Research and the Program of the ICD:

Seperation and isolation of subcellular-sized particles will be of importance for the biophysics and biochemistry of membrane complexes and other membrane component structures. For example, membrane trafficking is involved in fertilization, synaptic transmission, and hormone release. Electrophoretic purification of exocytotic and endocytotic vesicles will further our understanding of the steps involved in membrane trafficking and is thus important for our understanding of such diverse human maladies as infertility, neuronal and endocrine dysfunction, and diabetes.

Major Findings:

Vesicles from homogenates prepared from unfertilized and fertilized sea urchin eggs were separated by analytical CZE and preparative FFE and revealed three major fractions. These fractions were characterized by spectroscopy and DIC microscopy to reveal vesicles of different morphology.

Proposed Course:

We have applied electrophoretic methods (analytical CZE and preparative FFE) to separate sea urchin egg vesicles. We plan to maximize the separation by the inclusion of polymers. We will then characterize the vesicles in these fractions by SDS-PAGE. We then plan to compare the patterns of vesicle separation at various times after fertilization. Finally, we will determine if any of these isolated fractions retain biological functionality.

Subproject (ii) - Isolation of microsome components on automated gel electrophoresis apparatus: Component identification, yield and purity. (Chen, N. and Chrambach, A.)

Objective:

Previous studies have demonstrated rat liver microsome components by CZE in polymer solutions (Radko et al., 1995) and an increase in the number of such components under the conditions of fusion. Objective of the present study was to isolate the 3 major components existing in the absence of fusion conditions, to characterize them as microsome or its derivatives and to determine the yield of isolation and the purity of product.

Methods Employed:

The microsome sample was fluorescently labeled and subjected to agarose gel electrophoresis in the automated HPGE-1000 apparatus, using consecutive electroelution steps of 4 min duration twice for each of the 3 microsome components, providing 100 ul fractions in near-quantitative yield. These were dialyzed against volatile buffer and lyophilized. Purity (protein/weight) of the major component was close to 100%, and decreased to 70% for the least abundant component. SDS-polyacrylamide gel electrophoresis patterns of all 3 components were indistinguishable from those of the original microsome preparation, showing that the differences between the components resided in elements other than their major component proteins.

Major Findings:

An attempt was made to increase the resolution between microsome components by increase in field strength. This was achieved to a value of 80 V/cm by replacing the buffer with isoelectric histidine, pH 7.6 (Chen, N. and Chrambach, A., 1996). The best resolving polymer medium was selected by comparing the performance of agarose gel, agarose solution and laterally aggregated polyacrylamide gel. Since the 3 media gave similar results, agarose gel was selected as the most convenient medium compatible with the automated recovery procedure (Zakharov et al., 1995). Three major components were identified. Their SDS-polypeptide patterns were indistinguishably those of the microsome. Recovery in 1 or 2 electroelution steps for all 3 was near-quantitative. Protein of the main component constituted 70% of the weight of each component, similar to the protein content of the whole microsome preparation; thus no significant degree of impurity was found in the major component. The two minor components exhibited up to 30% non-proteinaceous impurities.

Proposed Course of the Project:

To build a generally applicable gel electrophoretic procedure for particle isolation a method for purifying the isolated components from polymeric impurities derived from the gel is still needed. Although the present dominant microsome component lacked detectable amounts of such impurities, other components, similarly to other proteins isolated from various polyacrylamide gel media, have been shown to contain about 30% by weight of polymeric impurities (Nguyen and Chrambach, 1979). These impurities can be removed by porous media in the cold, taking advantage of the stiffness of polymer chains around 0 C (ibid.). A microdialysis chamber for separating polymeric impurities from electroeluates of 100-200 μl volume is under construction.

Significance to Biomedical Research and the Program of the ICD:

Automated isolation of subcellular-sized particles will be of importance for the biophysics and biochemistry of membrane complexes and other membrane component structures.

Publications:

Vogel SS, Beushausen S, Lester DS. Application of a membrane fusion assay for rapid drug screening. Pharmaceut Res, 1995;12:1417-1422.

Vogel SS, Blank PS, Zimmerberg J. Poisson distributed active fusion complexes underlie the control of the rate and extent of exocytosis by calcium. J Cell Biol, 1996, in press.

Whalley T, Terasaki M, Cho M-S, Vogel SS. Direct membrane retrieval into large vesicles after exocytosis in sea urchin eggs. J Cell Biol 1995;131:1183-1192.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00171-20 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Electrophoretic Methodology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Chrambach	Head	SMA, LTPB, NICHD
Others:	S. Radko	Visiting Fellow	SMA, LTPB, NICHD
	N. Chen	Visiting Fellow	SMA, LTPB, NICHD
	G. Weiss	Collaborator	DCRT, NIH
	H. Sokoloff	Collaborator	DCRT, NIH
	P. Calabrese	Collaborator	DCRT, NIH
	H. Chang	Collaborator	Univ. Kentucky
	J. Sullivan	Collaborator	BEIB, NIH

COOPERATING UNITS *(if any)*

DCRT (G.H. Weiss, H. Sokoloff, P. Calabrese); University of Kentucky (H.-T. Chang)

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Macromolecular Analysis

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The methodological work of the year served the purpose of adaptation of originally macromolecular techniques to the separations of subcellular particles and membrane complexes. Theoretical interpretation of protein electrophoresis data led to the important insight in this regard that zone dispersion, a key element of resolving power, related to interactions with either gel fibers or walls of the vessel at capillary distances. That insight guided the development of a preparative method for large particles. Resolving power of separations was also augmented by the finding that power supply interrupted by rest periods allowing for Joule heat dissipation yielded at least a doubling of field strength, and surprisingly, that a derivatized, highly soluble agarose yielded a 4 to 5 fold increase in resolution over the conventional medium for protein separations, polyacrylamide. Automated instrumentation originally designed for gel electrophoresis had been shown last year to be applicable to large particles, replacing gels by concentrated polymer solutions. This technique has now been transformed into a preparative one for subcellular-sized particles. The wide applicability of the automated gel electrophoresis technique has also been advanced during the previous year by demonstration of its applicability to native, non-fluorescently labeled, particles. The originally poor detection sensitivity of that method has now been greatly improved by use of the fluorescence enhancer, salicylate, to control and maximize the level of the baseline for detection by "fluorescence reduction".

Project Description:

The aim of the project is the development of optimally resolving automated methods for the electrophoretic separation of macromolecules and subcellular particles. Since these species differ predominantly in size and shape (conformation), this requires selection of optimally resolving polymer type, concentration, field strength and migration time for two components in a particular size range. To be effective, this selection needs to be made on the basis of appropriate theory and models, with subsequent application of computer programs embedding such theory in conjunction with a data base of mobility and band dispersion values, provided by automated apparatus and capillary apparatus as a function of migration time. Of the greatest practical importance is the preparative capacity of electrophoresis in gels and polymer solutions. Automated apparatus allows for the monitoring of recovery steps to attain near-quantitative recoveries from gels and of subcellular-sized particles from polymer solutions. However, automated preparative electrophoresis still needs to be adapted to biological applications.

Objective:

This year's work aimed i) at the exploitation of the capacity for band width and shape determination and macromolecular isolations of the automated gel electrophoresis apparatus with intermittent fluorescence scanning of the migration path, and ii) at subcellular-sized particle separations by means of free-flow electrophoresis apparatus. i) Availability of the band profile was to be used to numerically evaluate resolution, the central parameter of separation science, for the different electrophoretic separation methods and media providing the "molecular sieve", and to evaluate the mechanisms of band dispersion and retardation operative in those media. The preparative application of the apparatus which uniquely allows for obtaining quantitative recovery through a sequential electroelution procedure was to be extended to subcellular-sized particles. ii) An attempt is made to widen the preparative potential of free-flow electrophoresis to include size separations of subcellular-sized particles.

Subproject (i-1) - Application of automated gel electrophoresis apparatus to non-fluorescent samples (Chen, N., Yarmola, E., Yi, D. and Chrambach, A.).

Objective:

A major handicap to the wide acceptance of automated gel electrophoresis apparatus with intermittent scanning of fluorescence has been the requirement for fluorescent labeling of non-fluorescing compounds. Such labeling not only is likely to modify the conformation of the molecule to an unknown degree, it also decreases the net surface charge and therefore the mobility in direct relation to the degree of labeling (Zakharov et al., 1995). A previous method of fluorescence detection of unlabeled compounds used an uncharged fluorescing agent (umbelliferone) in the gel, but appeared undesirable in view of the possibility of chemical interactions with that agent.

Major Findings:

Interposition of fluorescent yellow paper between the electrophoresis cell and the underlying mirror of the HPGE-1000 apparatus raises the fluorescent background of the gel scan to the top of the scan recording. Since the range of excitation wavelengths (280-360 nm) coincides with the absorbance peak of proteins at 280 nm, the intensity of the incident excitatory light is reduced by the absorbance of a protein, and the resulting fluorescence reduction produces an inverted band by which the unlabeled protein is detected. At low protein concentrations, the sensitivity of protein detection by fluorescence reduction is reduced by only a factor of 2 compared with that of fluorescence detection. At high protein concentrations, the sensitivity relative to fluorescence is reduced by a factor of up to 20. The detection sensitivity of the fluorescence reduction can be increased by impregnating the yellow paper with the fluorescence enhancer, Na-salicylate.

Proposed Course:

Exploiting the fact that the sensitivity of protein detection by fluorescence reduction is a linear function of protein concentration and that by fluorescence a logarithmic function, and considering the effect of varying the fluorescence spectrum (color and intensity) of the paper, the protein concentration range (depending on the extinction coefficient) yielding maximal detection sensitivity will be determined.

Significance to Biomedical Research and the Program of the ICD:

Detection of fluorescently unlabeled proteins by "fluorescence reduction" extends the benefits of automated gel electrophoresis apparatus (speed, labor-saving, quantitative band identification, pattern storage and preparativeness) to the native, functional proteins relevant to biochemistry.

Subproject (i-2) - Increased signal/noise ratio (detection sensitivity) by elimination of a mirror reflecting incident excitation light onto the migration path (Yarmola, E. and Chrambach, A.).

Objective:

The hypothesis underlying this project is that the increment in excitation light intensity produced by placing a mirror under the gel is outweighed by the increase in background noise which results in a decreased signal/noise ratio, requires larger sample loads and therefore produces an increased bandwidth and decreased resolution. Therefore, our objective was to increase the signal/noise ratio (detection sensitivity) of the HPGE-1000 apparatus by elimination of a mirror under the gel and consequent reduction of noise level. Increased detection sensitivity should allow for decreased sample load, and therefore decreased bandwidth and increased resolution.

Major Findings:

In a representative application of automated gel electrophoresis to proteins, elimination of the mirror below the gel tray increased the sensitivity of fluorescence detection (peak amplitude/average noise amplitude) by a factor of 6.3. That increase in detection sensitivity allows for lowered sample loads by which the band width is reduced and resolution is increased.

Proposed Course:

A instrumental modification eliminating or blackening the mirror under the gel of the HPGE-1000 apparatus is indicated.

Significance to Biomedical Research and the Program of the ICD:

The improvement of the detection sensitivity and resolving power of automated gel electrophoresis with scanning of fluorescence is of general interest for the ubiquitous applications of gel electrophoresis for biologically and medically relevant separations.

Subproject (i-3) - Increased resolution by periodic discontinuation of the electric field (Chen, N. and Chrambach, A.).

Objective:

The Joule heat dissipation capacity of gel electrophoresis apparatus is usually poor. That of automated apparatus extends to about 20 Watts, limiting the field strength applicable to that apparatus to approximately 15 V/cm with gels of 3 mm thickness, or 45 V/cm with those of 0.5 mm thickness (Chang, H.-T. and Chrambach, A., 1996). The value was increased to 80 V/cm by use of isoelectric histidine as the gel buffer (Chen, N. and Chrambach, A., 1996). Since resolution increases with field strength, the present study aimed at further increasing it, using a pulsed power supply to provide periodic cooling of the gel.

Major Findings:

Resolution between two representative proteins at 80 V/cm is doubled when 10 s of electrophoresis are periodically interspersed by 120 s in the absence of an electric field. The field strength could not be increased beyond that value since the limit of power supply is 1,000 V in the HPGE-1000 apparatus; but the doubling of resolution suggests that with a 2-3,000 V power supply (that would require rewiring of the instrument !) field strengths in the hundreds of V/cm could be achieved, similar to CZE, and with the same effect of raising the resolving capacity.

Proposed Course:

The results of the project suggest that gel electrophoretic separations in general should be conducted with pulsed interruption of the power supply whenever the advantage of resolution is not nullified by lengthened migration times.

Subproject (ii-1) - Prediction of protein retardation on the basis of the scaling theory (Radko, S. P. and Chrambach, A.).

Objective:

Previous prediction of the retardation of rigid spherical particles (proteins and charged polystyrenes) in gel electrophoresis has been based on the model of a ball penetrating a random inert network of fibers (the Ogston model). Thus, the model has been a purely geometric one, divorced from the real properties of polymer fibers. The present study aimed at correcting that situation by measuring retardation in the simplest case, that of proteins with Stokes radii of 2 - 5 nm (i.e. Mr 50 - 500 kDa), during elctrophoresis in polymer solution, and attempting to account for that retardation by the predictions of the scaling theory of polymer physics as to the properties of semi-dilute polymer solutions.

Major Findings:

The retardation of proteins in polyethylene glycol (Mr 0.2 - 8 million) solutions measured in CZE scales as the 0.75th power of polymer concentration and is independent of the Mr of the polymer, as predicted by the scaling theory for semi-dilute polymer solutions. Thus, the screening length, an essential parameter descriptive of semidilute polymer solutions, is responsible for molecular sieving (size dependent retardation).

Proposed Course:

To establish a comprehensive theory of retardation of rigid spherical particles in polymer solutions, it will be necessary to investigate retardation of larger particles, up to radii of 1 or more micra, in polymer solutions at different field strengths.

Significance to Biomedical Researchand the Program of the ICD:

A comprehensive theory of retardation in polymer solutions, derived from the experimentally supported theory of polymer physics, would allow for the prediction of molecular sieving over the wide particle size range of biochemistry.

Subproject (ii-2) - Retardation of particles with R>20 nm as a function of shear rate (Radko, S. P. and Chrambach, A.).

Objective:

Previously, the retardation of spherical particles by gels or polymer solutions has been assumed to be a function (linear, biphasic or undulating) of particle size (radius, R) and polymer properties only. Since for a particle with R>20 nm that relationship was not monotonic, particle size in that range could not be derived from retardation. The present study aimed at establishing a monotonic relationship to restore this possibility.

Major Findings:

Up to R of about 20 nm, retardation and particle radius are related near-linearly. When $R < 15$ nm, this relation is independent of field strength. When $R > 20$ nm, retardation and particle size relate in a complex undulating fashion, and in dependence on field strength. However, it was found that a simple relationship exists between retardation (at a particular polymer concentration) and shear rate, defined as (mobility x field strength)/R.

Proposed Course:

A method still needs to be found to derive R from shear rate for the entire R-range of interest. That will involve an attempt to narrow down or abolish the flat segment of the plot of retardation vs shear rate characteristic for low shear rates, by selecting an appropriate polymer.

Significance to Biomedical Research and the Program of the ICD:

For subcellular-sized particles, the finding of a monotonic decrease of retardation with shear rate opens up the possibility to derive their size from CZE data (at a given mobility at anyone polymer concentration and field strength).

Subproject (ii-3) - Band width and asymmetry of particles with $3<R<100$ nm in capillary zone electrophoresis in presence of polymers (Radko, S. P. and Chrambach, A.).

Objective:

Peak asymmetry provides information concerning the mechanism of peak spreading and resolution as a function of time and polymer concentration (the "Giddings-Weiss model", Weiss et al., 1996). Peak asymmetry was measured in CZE in polymer solution over a wide particle size range ($R = 3 - 100$ nm) with the aim of elucidating the sieving mechanism in polymer solution in the manner previously applied to gels.

Major Findings:

Band width and asymmetry are proportional. Both increase with polymer (polyacrylamide, 5 million Mr) concentration in the size range of proteins ($R < 7$ nm) independently of field strength, but vary in concave relation with polymer concentration and in dependence on field strength for larger particles (up to $R = 100$ nm). The asymmetry behavior in the smaller protein size range can be accounted for by the Giddings-Weiss model; that in the larger size range remains unaccounted for mechanistically.

Proposed Course:

Peak asymmetry will have to be measured over a wider particle size range, with different polymer sizes and types of polymer, and varying the field strength, to be able to widen the data base in support of the Giddings-Weiss model, and thereby widen its predictive range.

Significance to Biomedical Research and the Program of the ICD:

The expected predictability of resolution would upgrade the efficiency of size dependent separations in biochemistry.

Subproject (iii-1) - Dispersion and diffusion in gel electrophoresis (Yarmola, E., Sokoloff, H. and Chrambach, A.).

Objective:

It is widely assumed that bands in gel electrophoresis spread by diffusion with increasing migration time. However, previous studies on DNA (Yarmola, E. and Chrambach, A., 1995) and phycoerythrin (Chen, N., unpublished data) had shown that macromolecules don't diffuse appreciably in gels in absence of a field. Moreover, the deviation from Gaussian peak shape of phycoerythrin and conalbumin had suggested that the entanglement of the particle with the gel fiber, not diffusion, is predominantly responsible for band asymmetry, and therefore band spreading, in gels (Weiss et al., 1996). Therefore, the aim of the present study was to solidly establish that diffusion was not a significant element of macromolecular band spreading in gels.

Major Findings:

DNA of 1, 2 and 3 kb length were subjected to gel electrophoresis with measurement of band spreading. The power supply was turned off, and band spreading in the absence of an electric field was measured. Expressing the rate of band spreading as an electrophoretic dispersion coefficient and a diffusion coefficient in absence of a field, respectively, the ratio of the two was found to be 5/1, 15/1 and 45/1 for 1, 2 and 3 kb DNA respectively.

Proposed Course:

The study made it clear that diffusion was not a major source of macromolecular band spreading in gels. This fact requires a new model for band dispersion to allow one to predict band spreading, and thus resolution in gels (see section iii-2 below).

Significance to Biomedical Research and the Program of the ICD:

The findings of the study overcome a fundamental inaccuracy in the way resolution in gel electrophoresis has been determined, i.e. the assumption that band spreading is governed by diffusion.

Subproject (iii-2) - A model predicting band dispersion in gel electrophoresis (Yarmola, E., Calabrese, P., Chrambach, A., and Weiss, G. H.).

Objective:

To construct a model predicting band spreading in gels, a systematic study of the spreading of phycoerythrin bands as a function of time, load, gel concentration, ionic strength and field strength was carried out.

Major Findings:

Phycoerythrin band variance varies non-linearly (close to parabolically) with time of electrophoretic dispersion and linearly with time of diffusion in the absence of a field. Peak area decreases slightly in proportion to migration time. Since the area is proportional to load, the decline of area with migration time increases with increasing protein load. Variance of electrophoretic dispersion increases as the field strength is lowered, while band spreading is independent of field strength during diffusion. Log (peak amplitude) decreases with log (migration time) linearly. Resolution increases with migration time to a constant plateau value. A mathematical model consistent with these data was devised which allows one to predict dispersion in gel electrophoresis.

Proposed Course:

Although we are now able to predict the extent of dispersion for a rigid spherical protein, the mechanism of band spreading as a function of conductance, field strength, viscosity, sample load, temperature and gel concentration remains unknown. It will be necessary to investigate each of these parameters systematically and solve simultaneously the functions governing their interrelations, to be able to account for the elements responsible for the observed and predicted band spreading.

Significance to Biomedical Research and the Program of the ICD:

Band spreading, its accurate prediction and a knowledge of the elements responsible for it are important for the predicting and controlling resolution in analytical and preparative gel electrophoresis.

Subproject (iv) - Resolving power of gel media (Chen, N. and Chrambach, A.).

Objective:

It is widely assumed that polyacrylamide gels excel in their resolving capacity for proteins over agarose gels. Also, the commercial literature suggests that some agaroses such as MetaPhor-XR exhibit a greater resolving capacity for proteins than other agarose types. The possibility to quantitate resolution by use of scanning apparatus as a function of migration time and gel concentration opened up a way to test those two assumptions.

Major Findings:

Measurements of migration distances and band widths on two representative proteins show that SeaPrep agarose, 10% derivatized with hydroxyethyl residues and therefore highly soluble, exceeds polyacrylamide in resolving power for proteins by a factor of 4-5. MetaPhor-XR, Gold, SeaKem GTG have less resolving capacity than SeaPrep.

Proposed Course:

The most widely used polyacrylamide gels are those used for analysis of SDS-polypeptides in a single dimension or in the second dimension. The effectiveness of SeaPrep agarose gels or pore gradient gels made from it will be tested in those applications.

Significance to Biomedical Research and the Program of the ICD:

Substitution of polyacrylamide by agarose should result in improved reproducibility of mobilities, since the former is the product of a complex free radical polymerization reaction, the latter a product of simple thermally controlled gelation.

Subproject (v) - Band area dynamics during migration (Zakharov, S. F., Kwo, S.H., Sokoloff, H., Chang, H.-T., Radko, S.P., and Chrambach, A.).

Objective:

Gel electrophoresis patterns obtained as a function of migration time by use of scanning apparatus give the appearance of declining band areas. Since constancy of band areas with migration time is a prerequisite for measuring resolution, it needed to be ascertained for a variety of proteins, both naturally fluorescent and fluorescein carboxylate labeled.

Major Findings:

Fluorescent protein band area is nearly constant with migration time; a slight decline appears to be due to photochemical destruction of the fluorophore. Protein band area detected by fluorescence reduction (Subproject i-1) is constant with migration time. Band area decreases with gel concentration. These results rest on an accurate determination of the baseline which varies with migration distance. Ascending and descending tails of the peak are a constant fraction of the total area of the peak independently of migration distance.

Proposed Course:

Having shown that band area is nearly constant during migration, the band width measurement is validated. The dependence of area on gel concentration is of mechanistic interest and deserves further work.

Significance to Biomedical Research and the Program of the ICD:

The demonstration of area constancy with migration time satisfies a necessary condition for the determination of bandwidth and resolution and therefore is of general importance for the evaluation, prediction and control of gel electrophoretic separations.

Subproject (vi) - Restoration of automated procedure for the isolation of particles from concentrated polymer solutions, using the HPGE-1000 apparatus (Chang, H. T., Chen, N., Sullivan, J. V. and Chrambach, A.).

Objective:

Last year, we reported the isolation of a microsomal electrophoretic fraction from a concentrated polymer solution using the HPGE-1000 apparatus (Chang, H.-T. and Chrambach, A, 1995). Although the intermittent scanning during separation was automated, the isolation was not: After separation, the band was manually detected by UV-lamp and manually withdrawn by syringe. The present project aimed at restoring automation to that procedure.

Major findings:

Automation was restored to the isolation procedure from concentrated polymer solutions by 3 instrumental modifications on the HPGE-1000 apparatus: i) A sample cup insert holding a thin Teflon tube in the cup was constructed and the arms holding the sample cup in the apparatus were elongated so that the bottom edge of the cup reached 2 to 2.5 mm into the polymer solution of 3 mm height; ii) the syringe by which the band of interest is withdrawn was connected to the insert by a thin Teflon tube of sufficient length to allow the sample cup any position along the migration path; iii) a hole was drilled into the cover of the HPGE-1000 apparatus to allow for the tube connection between syringe and sample cup; the hole was covered by mounting the syringe solidly in a frame on top of the instrument cover. These instrumental modifications allowed one to position th sample cup over the band of interest automatically and to withdraw the band by syringe while the sample cup was automatically placed into the desired position.

Proposed Course:

The automated isolation procedure, using electrophoresis in concentrated polymer solutions, will be applied to subcellular particles, such as sea urchin egg vesicles isolated by FFE [see the companion project, subproject (I)], to obtain fractions of these particles differing in size and shape.

Significance to Biomedical Research and the Program of the ICD:

A size fractionation of subcellular particles is not available at this time. The procedure described above should fill that void. It seems likely that subcellular particles differ in size and shape to a higher degree than in surface net charge/mass ratios, and that therefore the procedure should allow one to arrive at a more effective fractionation of such particles than available from procedures which exploit free mobility differences..

Publications:

Aldroubi A, Zakharov SF, Chrambach A. A computer program for predicting recovery of SDS-protein in the automated HPGE-1000 apparatus. Appl Theor Electrophoresis, 1995;5:31-34.

Aldroubi A, Chang HT, Zakharov SF, Chrambach A. Determination of optimally resolving gel concentration and migration time (path) in gel electrophoresis. Anal Biochem, 1995;231:432-436.

Chang HT, Chrambach A. Horizontal gel electrophoresis with sample volumes up to 1.5 ml, using a discontinuous buffer system and automated apparatus. Electrophoresis, 1995;16:952-957.

Chang HT, Chrambach A. Application of gels of 0.5 mm thickness to electrophoresis in the automated HPGE-1000 apparatus: Improved resolution. Electrophoresis, 1996;17:80-83.

Chang HT, Chrambach A. Feasibility of electrophoresis of a subcellular-sized particle in polymer solutions, using automated horizontal gel apparatus. Appl Theor Electrophoresis 1995;5:73-77.

Chang HT, Zakharov SF, Chrambach, A. Separation and isolation of subcellular-sized particles by electrophoresis in polymer solution, using the commercial scanning apparatus. Electrophoresis, 1996;17:776-780.

Chen N, Chrambach A. Enhanced field strength and resolution in gel electrophoresis upon substitution of buffer by histidine at its pI. Electrophoresis, 1996;17:699-703.

Chen N, Chrambach A. Improved resolution in the gel electrophoresis of proteins by a periodically interrupted electric field. J Biochem Biophys Methods, 1996, in press.

Chen N, Chrambach A. Application of the commercial gel electrophoresis apparatus with intermittent fluorescence scanning to a non-fluorescing protein. Anal Biochem, 1996, in press.

Chrambach, A. Quantitative and automated electrophoresis in sieving media: Past, present and future. Electrophoresis, 1996;17:454-464.

Radko SP, Chrambach A. Molecular sieving by polymer solutions: Dependence on particle and polymer size and dispersion, independence of polymer entanglement. Appl Theor Electrophoresis, 1995;5:79-88.

Radko SP, Chrambach A. Mechanisms of retardation of rigid spherical particles with 3 to 1,085 nm radius in capillary electrophoresis, using buffered polyacrylamide (Mr=5 million) solutions. Electrophoresis, 1996, in press.

Radko SP, Chrambach A. Parallelism between width and asymmetry of peaks of rigid, spherical particles in CZE using polymer solutions. Appl Theor Electrophoresis, 1996, in press.

Radko SP, Chrambach A. Electrophoresis in polymer solutions: Mechanisms of "molecular sieving". J Phys Chem, 1996, in press.

Weiss GH, Garner MM, Yarmola E, Bocek P, Chrambach A. A comparison of the resolution of DNA fragments between agarose gel and capillary zone electrophoresis in agarose solutions. Electrophoresis, 1995;16:1345-1353.

Weiss GH, Sokoloff H, Zakharov S F, Chrambach A. Interpretation of electrophoretic band shapes by a partition chromatographic model. Electrophoresis, 1996, in press.

Yarmola E, Sokoloff H, Chrambach A. The relative contributions of dispersion and diffusion to band spreading (resolution) in gel electrophoresis as a function of DNA length. Electrophoresis, 1996, in press.

Zakharov SF, Garner MM, Chrambach, A. Recovery of SDS-protein and DNA using commercial automated gel electrophoresis apparatus. Appl Theor Electrophoresis, 1995;5:25-30.

Zakharov SF, Chang HT, Chrambach, A. Reproducibility of mobility in gel electrophoresis. Electrophoresis, 1996;17:84-90.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01400-14 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Dynamics of the Growth and development of Bone

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Yergey	Head	SMAMS, LTPB, NICHD
Others:	N. Vieira	Biologist	SMAMS, LTPB, NICHD

COOPERATING UNITS *(if any)*

Human Genetics Branch, NICHD (J. Marini); Nemours Children's Clinic, Jacksonville, Florida (N. Mauras)

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Metabolic Analysis and Mass Spectrometry

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ © Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The effect of recombinant human growth hormone, hGH, treatment on Ca metabolism and growth is being investigated in patients with Osteogenesis Imperfecta Type III in collaboration with J. Marini, HDB. Fractional absorption of dietary calcium and the rate of Ca distribution within the body is studied in these patients before and after treatment with hGH. Preliminary results of from 5 subjects show an apparent decrease in fractional absorption of dietary calcium (0.29 ± 0.19 pre treatment vs 0.14 ± 0.11 post treatment). At the same time an increase in both the whole body exchangeable pool (TEP = 265 ± 95mg/kg pre treatment vs 673 ± 616mg/kg post treatment) and rate of calcium accretion to bone (Vo+ = 94 ± 29mg/kg/d pre treatment vs 142 ± 75mg/kg/d post treatment) was observed. While suggestive, the variance in the mean values of these results is too great to be significant at the present time. Further studies to confirm these observations are underway.

Project Description:

Objectives:

The principal objective of this work is to determine the dynamics by which calcium is accreted to and removed from bone during human growth and development. The investigations conducted in this project have represented an attempt to assemble congruent, or at least convergent, views of calcium dynamics from studies of intact human subjects. To date this objective has been met by studies of absorption and distribution of calcium in healthy infants and children, in children with diseases suspected of altering calcium metabolism and in healthy women in altered states of calcium metabolism such as pregnancy and lactation.

Methods Employed:

The principal methodology used employs stable isotopes of calcium as tracers for the entrance and distribution of calcium in the body. Determination of isotope balances allows the calculation of important parameters of calcium metabolism, principally the rates of absorption and excretion. Fitting of mathematical models to the time-dependence of the disappearance of the intravenous isotope, when coupled with the balance data, leads to the calculation of the rates of calcium accretion to and resorption from bone. Thermal ionization mass spectrometric measurements of calcium isotope ratios of material isolated from blood and excreta are the observations obtained from these studies. The mathematical models which are fit to these observations represent ongoing attempts to characterize the dynamics of bone remodeling.

Major Findings:

1.The effect of recombinant human growth hormone, hGH, treatment on Ca metabolism and growth is continuing to be investigated in patients with Osteogenesis Imperfecta Type III in collaboration with J. Marini, HDB. These patients are studied before and after treatment with hGH. Preliminary results of calcium metabolism/distribution studies in 5 subjects studied pre and post treatment show an apparent decrease in fractional absorption of dietary calcium (0.29±0.19 pre treatment vs 0.14±0.11 post treatment). At the same time an increase in both the whole body exchangeable pool (TEP = 265±95mg/kg pre treatment vs 673±616mg/kg post treatment) and rate of calcium accretion to bone (Vo+ = 94±29mg/kg/d pretreatment vs 142±75mg/kg/d post treatment) was observed. While suggestive of possibly important consequences, the variance in the mean values of these results is too great to be significant at the present time. We are conducting further studies to confirm these observations and are in the process of analyzing 6 additional studies undertaken in the past year.

Significance to Biomedical Research and the Program of the ICD:

An overall understanding of the developmental aspects of calcium absorption, excretion and distribution to bone as a function of development in humans is emerging as a consequence of these studies. Our results can begin to be used to interpret abnormalities of calcium metabolism in a manner that can be therapeutically useful.

Proposed Course:

The nature of this project has become progressively more clinical in what is fundamentally a physical chemistry oriented section. As a consequence, the section will move away from this area of research by completing current projects, by not undertaking new collaborative efforts and, to the extent possible, by finding physical-measurement-oriented clinical colleagues interested in continuing current lines of investigation.

Publications:

Goans RE, Weiss GH, Vieira NE, Sidbury JB, Abrams SA, Yergey AL. Calcium Kinetics in Glycogen Storage Disease. Calcif Tiss Int, 1996, in press.

Kalkwarf HJ, Specker BL, Heubi JE, Vieira NE, Yergey AL. Intestinal Calcium Absorption of Women during Lactation and after Weaning, Am J Clin Nutr, 1996;63:526-531.

Koumenis IL, Yergey AL, Abrams SA, SN Deming, Hutchins W, Vestal ML. Quantitation of Metal Isotope Ratios by Laser Desorption Time-of-Flight Mass Spectrometry. Anal Chem, 1995;67:4557-4564.

Vieira NE, Yergey AL. Extraction of Serum and Urine Calcium Using Ion Exchange Membrane Filters for Isotope Enrichment Analysis Using Thermal Ionization Mass Spectrometry. Anal Chem, 1995;67:4217-4219.

Yergey AL, Vieira NE, Abrams SA, Marini J, Goans RE. Use of Stable Isotopic Tracers in Studies of Whole Body Calcium Metabolism. Connective Tiss Res, 1995;31:291-293.

Yergey AL. Stable Isotopic Tracers in Mineral Metabolism. J Nutr, 1996;126:355S-359S.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01409-11 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Membrane Remodeling During Viral-Induced Syncytia Formation and Parasitic Invasion of Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Zimmerberg	Chief	LTPB, NICHD
Others:	I. Plonsky	Visiting Fellow	LTPB, NICHD
	E. Suss-Toby	Visiting Fellow	LTPB, NICHD
	D. Kingsley	IRTA Fellow	LTPB, NICHD

COOPERATING UNITS *(if any)*

Laboratory of Parasitic Diseases, NIAID (Gary Ward); Cornell University, (Gary Blizzard)

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Membrane and Cellular Biophysics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.75	2.75	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are continuing our work on fusion pores, the first detectable intermediate in biological fusion. We previously described pores induced by the *influenza* envelope protein hemagglutinin (HA). We now report features of fusion pores induced by another viral protein, baculovirus GP64. Unlike for HA, the GP64-induced fusion pore on Sf9 cells has a large initial conductance, does not flicker, and forms much faster with kinetics which fits best to a parallel model with many independent elements. To investigate if features of fusion pores are determined only by fusion proteins or they could be influenced by other viral envelope proteins or characteristics of target membranes, we express GP64 in a stably transfected cell line (Op-1D) and study fusion of Op-1D cells with erythrocytes. Red blood cells were previously used as target cells for research on HA-induced fusion. Our preliminary analysis showed that other baculovirus envelope proteins or features of target membrane unlikely interfere with initial pore conductance but rather have an impact on fusion pore kinetics.

Another pore figures in membrane remodelling, the fission pore. Most intracellular pathogens avoid lysing their host cells during invasion by wrapping themselves in a vacuolar membrane. This parasitophorous vacuole membrane (PVM) is often retained, serving as a critical transport interface between the parasite and the host cell cytoplasm. To test whether the PVM formed by the parasite *Toxoplasma gondii* is derived from host cell membrane or from lipids secreted by the parasite, we used time-resolved capacitance measurements and video microscopy to assay host cell surface area during invasion. We observed no significant change in host cell surface area during PVM formation, demonstrating that the PVM consists primarily of invaginated host cell membrane. Pinching off of the PVM from the host cell membrane occurred after an unexpected delay (34-305 s) and was seen as a 0.219 +/- 0.006 pF drop in capacitance, which corresponds well to the predicted surface area of the entire PVM (30-33 m^2). The formation and closure of a fission pore connecting the extracellular medium and the vacuolar space was detected as the PVM pinched off. This final stage of parasite entry was accomplished without any breach in cell membrane integrity.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

To investigate if the fine structure of fusion proteins is only one determinant of features of fusion pores (FPs), two FPs with distinct characteristics should ideally be compared under the same conditions. FP phenotype could be influenced by different factors. First, features of host and target membranes. To date, only the hemagglutinin (HA)-induced FP was rigorously described in a system where HA was expressed in stably transfected fibroblasts (host cells); erythrocytes were used as target cells. In our previous study, FPs between infected Sf9 cells were explored. Both cells bear fusion, as well as other viral proteins. The host and a target membranes are the same. To investigate whether other envelope viral proteins or features of a target membrane may interfere with FPs, a fusion of stably transfected, GP64-expressing Sf9 cells with erythrocytes was studied.

T. gondii is an obligate intracellular parasite. During invasion, which is an active process distinctly different from phagocytosis, the parasite becomes surrounded by the PVM. The PVM pinches off from the host cell membrane at the end of invasion, to create a vacuole within which the parasite grows and replicates to continue the infection. As the interface between the parasite and the host cell, the PVM functions in metabolite uptake, nutrient transport, and protein trafficking. The origin of the PVM in cells infected with *Toxoplasma* and other Apicomplexan parasites is unknown, and has been the subject of considerable controversy. Two main models have been proposed to explain how the PVM is formed. The bilayer insertion model predicts a significant increase in surface area during PVM formation, followed by a drop in surface area as the PVM pinches off. The induced invagination model predicts no significant increase in surface area during PVM formation, followed by a drop in surface area as the PVM pinches off. Our goal was to perform experiments designed to directly test these predictions, using time-resolved capacitance measurements to measure host cell surface area while simultaneously monitoring invasion optically.

Methods employed:

Viral Fusion Pores

Stably transfected, GP64 expressing Op-1D cells were used for electrophysiological experiments. Fusion protein belonged to OpMNPV strain of *Baculoviridae*. Human erythrocytes were taken as target cells. FP conductance (G_p) and erythrocyte capacitance (C_{m2}) were calculated using the time-resolved admittance measurements technique. Adjustments of a phase angle ( ) and calculation of a scaling factor (T) were performed automatically as implemented in our laboratory-developed "Browse" software. G_p, C_{m2} were calculated as $G_p=(Re^2+Im^2)/ReT$, $C_{m2}=(Re^2+Im^2)/ImT$, where Re, Im are fusion-induced increments of real and imaginary parts of an electrical admittance. To trigger cell-cell fusion, highly buffered acid solution (pH 5.0) was ejected from a micropipette, placed at about one cell diameter aside a cell pair. The kinetics of fusion was studied by measuring waiting times $(_{to})$ between triggering and fusion pore appearance. Fusion pores which are characteristic for another strain of *Baculoviridae*, AcMNPV, were characterized earlier.

Host cells and parasites

COS-1 cells (ATCC CRL 1650) were cultured in DMEM complete medium. *Toxoplasma gondii* (RH) tachyzoites were cultured in human foreskin fibroblasts (ATCC CRL 1634). Spontaneously released parasites from a massively infected monolayer (moi>10) were passed twice through a 27G needle, filtered through a 10m PCTE filter (Poretics), spun for 5 min. at 1100 Xg, and resuspended in bath solution (see below).

Optical visualization of invasion

Experiments were performed on a Zeiss IM35 inverted microscope equipped with differential interference contrast (DIC) and epifluorescence optics. The parasite suspension was loaded into a beveled parasite delivery pipette (15 m opening), and delivered to the COS-1 cells using an Eppendorf 5242 micro injector. For the fluorescence invasion assay, anti-P30 monoclonal antibody (Biogenex) was conjugated with fluorescein as follows. Sodium bicarbonate (200 ml, 1M) and 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid, succinimidyl ester (100 ml, 10 mg/ml) were added sequentially to 1 ml (250 mg) of anti-P30, and incubated for 90 min. at 23° C. The reaction was stopped with hydroxylamine (100 ml, 1.5 M, pH 8.0), incubated 30 min. at 23° C, and passed through a PD-10 column (Pharmacia) equilibrated with phosphate buffered saline (PBS). The excluded fraction containing the conjugate (1.4 ml) was collected, sterile filtered, and stored at 4° C. To assay invasion using the conjugate, the microscope stage heater was turned off, and the bath solution was replaced with chilled (4° C) PBS containing 0.5% (wt./vol.) bovine serum albumin (PBS/BSA). After 4 min., this solution was replaced with 1.5 ml PBS/BSA containing 30 ml fluorescein-conjugated anti-P30, and incubated for 12 min. at 23° C. The dish was washed 4 times with chilled PBS/BSA (3 min., 3 ml each) and viewed with epifluorescence optics.

Electrophysiology.

The imaginary and real parts of the cell admittance were calculated from the output current of an EPC7 patch clamp amplifier (List-electronic) in the voltage clamp mode, using our own software for a digital lock-in amplifier

"Browse". The lock-in outputs represent the changes in capacitance (C) and AC conductance (G_{ac}) of the capacitor of a vacuole (C_v) in series with the conductance of a fission pore (G_p), as given by (20): $G_{ac} = ((C_v)^2/G_p)/ (1+(C_v/G_p)^2)$ and $C = (C_v)/(1+(C_v/G_p)^2$ where = 2 (frequency of applied sine wave). G_p was calculated from these equations as well as from the combined expression: $G_p = ((G_{ac})^2+(C^2))/ G_{ac}$.

Major Findings:

Theory of admittance measurements

For the Im-based calculation algorithm, a robust measurement of fusing membrane capacitance lags fusion, because the electrical access to new membrane is limited by a fusion pore. As far as G_p becomes negligible compare to an admittance of C_{m2}, an accurate value can be obtained. For our latest, Im/ Re-based algorithm, an accurate value of C_{m2} could be theoretically obtained even for small pores. In practice, when the FP is small, Im/ Re-based values of C_{m2} can still be smaller or higher than expected. We postulated that such deviations are explained by small errors in phase adjustment. C_{m2} can be restored by "off line" correction of : a correct phase angle corresponds to step-like shape of C_{m2}. An option for manual "off-line" correction of is implemented in our software ("Browse"). We also showed that such small errors in phase adjustment do not introduce inaccuracies in initial G_p values or has a negligible impact on the results of C_{m2} calculation when FP is large. The results of this theoretical analysis is especially important for the study of virus-induced fusion, where FP develops slower than in exocytosis.

Viral Fusion Pores

Eleven GP64-expressing, stably transfected cell lines were produced in collaboration with Dr. Blissard. They bear the OpMNPV strain GP64 (Op GP64). Cell lines were tested for their ability to form fusion pores in the time frame of a patch-clamp experiment; one of them (Op-1D) was suitable for time-resolved admittance measurements. In 66% of experiments (n=53), Op-1D/erythrocyte fusion occurred. Initial fusion pore never flicker; its conductance varied between 0.3-4.6 nS with a median of 1.1 nS. Erythrocyte membrane capacitance was equal to 1.1 0.2 pF (range: 0.8-1.5 pF), close to the expected value. Waiting times from pH delivery to FP appearance varied between 0.16-4.65 s with a median of 0.44 s. Using a Mann-Whitney statistical non-parametric test we compared Op-1D cell/erythrocyte to AcMNPV-infected Sf9 cell/Sf9 cell fusion. Our analysis showed that initial pores induced by two different baculoviruses' fusion proteins had the same initial conductance. Op-1D/erythrocyte fusion had significantly smaller $_{to}$ median value (0.44 *vs.* 0.63 s). For AcMNPV-infected Sf9/Sf9 fusion, the waiting time distribution fit best to a parallel model with many independent elements. The crux of the model is that after triggering, several independent identical elements move from non fusion-permissive to fusion-permissive positions. After all these transitions happen, fusion takes place. This approach is also applicable for Op-1D/erythrocyte fusion: the calculated rate constant of forward reaction is greater then in case of AcMNPV-infected fusion (14.5 *vs.* 6.7 s^{-1}).

Thus, fusion pores between stably transfected Sf9 Op GP64-expressing cells with erythrocytes were characterized. Our preliminary analysis was based on the comparison of FP which are characteristic for Op-1D/erythrocyte fusion *vs.* pores, observed for AcMNPV-infected Sf9 cells fusion. Our results are consistent with the idea that the target membrane and the presence of other envelope viral proteins are unlikely to interfere with the initial pore conductance but rather could have an impact on fusion pore kinetics.

Parasitic Fusion Pores

Parasites glide along the coverslip and surface of the cell at approximately 1 m/sec with a characteristic twisting motion. The gliding stops immediately prior to invasion, when the apical end of the parasite attaches to the host cell plasma membrane. Interestingly, when the apical end of the parasite first attaches to the host cell, a large transient in host cell membrane conductance (spike) is invariably observed (n>100). The nature and significance of the spike is currently under investigation; we used the time of the spike in this study to define the zero time of invasion. A constriction, readily visible with DIC optics, then develops at the apical end of the parasite and passes along the length of the parasite as it squeezes into the developing PVM. This constriction is the hallmark of an invading parasite. To independently confirm that a constricting parasite had indeed internalized, we developed an immunofluorescence assay in which free parasites and parasites bound to the external surface of the cell were brightly fluorescent, while internalized parasites were inaccessible to the antibody and were not stained.

The capacitance of the host cell did not increase during PVM formation. Two different methods were used to quantitate capacitance changes during PVM formation. By either method, host cell capacitance did not change (0.000 +/- 0.023 pF, s.e.m.; n=11). Since the capacitance change associated with pinching off of the PVM was found to be -0.219 pF, these data are clearly inconsistent (*t*-test, a=.005) with the hypothesis that the PVM is composed primarily of newly inserted bilayer, which would predict an increase in host cell capacitance during invasion of this magnitude. The mean change we measured by both methods of analysis was zero, and the data allow us to rule out (a =.05) any hypothesis in which there is a change of greater than 0.031 pF (method #1) or 0.041 pF (method #2), which corresponds to 14.2% or 18.5% of the total surface area of the PVM, respectively. Capacitance measures the net surface area, and while it is possible that the host cell tightly regulates its surface area and compensates for parasite-added lipids by taking up membrane as lipids are inserted there was no evidence for such regulation after a substantial loss of membrane, the pinching off of the PVM.

More than one third of the drops in capacitance (n=29) fluctuated. When a cell was invaded by more than one parasite, multiple capacitance drops were seen, all of approximately the same magnitude. If we model the parasite as two overlapping prolate ellipsoids with negligible separation of parasite and PVM, the calculated surface area of the PVM would be 30-33 mm^2. This yields a specific capacitance of the PVM of 0.0067-0.0073 pF/mm^2, within the range of biological membranes and remarkably close to measurements of solvent-free planar bilayer membranes. Surprisingly, pinching off of the PVM did not occur at the end of constriction (EOC); the time between EOC and the drop was on average 201 +/- 31 s (s.e.m, n=6) with an aspartate-based internal solution, 122 +/- 16 s (n=7) with a chloride-based internal solution, and 73 +/- 3 s (n=4) under PPR conditions.

Our measurement of the electrical parameters of the cell membrane during the entire process of invasion allowed us to detect the parasite-induced fission pore. A fission pore is the small aqueous connection between a forming endocytic vacuole (here, the parasitophorous vacuole) and the extracellular medium which forms and constricts to closure during the final stages of vacuole pinch off. A conceptually similar pore develops and widens during exocytosis, upon the formation of the first aqueous connection between the fusing exocytotic granule and the extracellular space. As a fission pore closes, it creates impedance to the free diffusion of ions between the vacuolar space and the extracellular fluid. The fission pore conductance was at first too large to be measured, and then declined to an initial measurable value. As it closed further, the pore conductance frequently lingered at conductances with mean values which were momentarily stationary, indicating one or more semistable intermediate stages prior to complete closure. Detected pore conductances ranged from 12 to 0.1 nS, a range which overlaps with that of molecular ionic channels. The capacitance fluctuations described above reflected a rapid relaxation from a semi-stable pore conductance to a larger conductance, followed by a second (or third) constriction and complete closure. Fission pore conductances calculated from both C and G_{ac} were in accord with the fission pore conductances calculated from C alone; no additional increase in cell conductance, which would indicate a breach in cell integrity, was detected.

This report represents the first application of electrophysiological methods to the study of host cell invasion during parasitic infection. The capacitance data show that while a small amount of parasite-derived material (0-18.5 % of the total surface area of the PVM) may be inserted into the host cell membrane during invasion, most if not all of the PVM is derived from the host cell plasma membrane. Specific proteins and lipids are thought to be transferred from the apical organelles of the parasite to the host cell during invasion, and might serve to induce the invagination or to modify the functional properties of the PVM. Our data are clearly inconsistent, however, with the idea that the PVM is composed primarily of lipids secreted by the parasite. They are also inconsistent with an earlier model in which the parasite enters directly into the host cell cytoplasm by disrupting a region of host plasma membrane, as this model would not predict the PVM-sized drops in host cell capacitance.

We have demonstrated an unexpected delay between the end of constriction and the pinching off of the PVM. This delay occurred even under perforated patch conditions, which minimize cytoplasmic washout. Preliminary experiments using fluorescent antibody against the parasite surface protein P-30 and fluorescent lipophilic probes suggest that prior to pinching off, the PVM is continuous with the host plasma membrane but its interior is inaccessible to extracellular soluble macromolecules. This is reminiscent of the constricted pit stage of receptor-mediated endocytosis, and may represent a period of time during which host and/or parasite factors playing a role in the pinching off of the PVM can be studied. Host cell plasma membrane proteins which might normally target an endocytic vacuole for fusion with the endolysosomal system are apparently somehow removed from the *Toxoplasma* PVM shortly after parasite internalization. The lag period may provide the parasite with an opportunity to return these unwanted targeting signals to the host plasma membrane prior to pinching off of the PVM.

Pinching off of a vacuole from the plasma membrane involves the formation of a neck through the bending of the continuous, unitary membrane which comprises and links the plasma and vacuolar membranes, followed by severe constriction (to molecular dimensions) of this neck and a fission event during which the neck breaks and two distinct membranes emerge from the one. The parasitic fission pore observed during PVM pinch off was in many respects indistinguishable from the endocytic fission pore seen during membrane retrieval following exocytosis in the pituitary nerve terminal. Both demonstrated intermediate semistable conductance phases, and both showed a smooth, continuous decline in conductance prior to final closure. Compared to membrane fusion, membrane fission appears to be a slow process and is characterized by a continuous narrowing of a small pore with no final jump, while in membrane fusion, a distinct jump in pore conductance is observed prior to pore growth. The reversals in pore narrowing we have observed in the parasitic fission pore are occasionally seen in exocytotic fusion pores, but not as abruptly as seen here. Furthermore, we have seen no evidence of reopening of a completely closed parasitic fission pore (analogous to exocytotic flicker). It is tempting to regard the rapid re-enlargement of the almost-closed fission pore as a reflection of the disaggregation of, or escape from, a putative proteinaceous constricting apparatus around the cytoplasmic neck of the pore.

Significance to Biomedical Research and the Program of the ICD:

The fusion pore is the object through which viruses infect cells; the fission pores is the object through which parasites infect cells. It is essential that we understand this structure at the highest level of resolution in order to design ways of stopping viral infection at the stage of cell entry. These studies are allowing us to experiment on the character of the proteins and membranes in order to determine which is dominant in fusion pore characteristics. *Toxoplasma gondii* is an important pathogen of humans, causing congenital birth defects when acute infection occurs during pregnancy, and severe encephalitis in immunocompromised persons. Toxoplasmic encephalitis has recently emerged as one of the most serious opportunistic infections associated with AIDS. Like other Apicomplexan parasites (including *Plasmodium*, the causative agent of malaria), Toxoplasma is an obligate intracellular parasite. When the PVM cannot pinch-off, invasion is arrested and the host cell lyses. Future studies of the parasitic fission pore may therefore yield new insights not only into the structure of the endocytic fission pore but also may help generate novel strategies to arrest infection.

Proposed Course:

An unambiguous answer to the question whether features of FPs depend on a target cell membrane requires the results of control experiments on Op-1D cell/Sf9 cell fusion. Expression of HA in Sf9 cells and study of HA as well as GP64-induced fusion pores will help us to answer the question if the fine structure of fusion proteins determines initial FPs. Chimerical forms of fusion proteins in baculovirus expression system could be useful as tools for finding of protein domains which are responsible for FP phenotype. Studies of the PVM will now focus on questions of how the invagination is induced, and how a vacuole derived from the host cell membrane can resist fusion with the endolysosomal system of the host cell.

Publications:

Zimmerberg J, Vogel S, Whalley T, Plonsky I, Sokoloff A, Chanturiya A, Chernomordik L. Intermediates in membrane fusion. Cold Spring Harbor Symposia on Quantitative Biology, 1995;Vol. LX:589-599.

Suss-Toby E, Zimmerberg J, Ward G. Toxoplasma invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore. Proc Nat'l Acad Sci USA, 1996;93:8413-8418.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01415-06 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Components and Kinetics in Exocytosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Zimmerberg	Chief	LTPB, NICHD
Others:	P. Blank	IRTA Fellow	LTPB, NICHD
	S. Vogel	Senior Staff fellow	LTPB, NICHD
	A. Sokoloff	Visiting Associate	LTPB, NICHD
	J. Coorssen	Visiting Fellow	LTPB, NICHD
	M. Tahara	Visiting Fellow	LTPB, NICHD
	T. Whalley	Visiting Fellow	LTPB, NICHD

COOPERATING UNITS *(if any)*

Stanford University, (Richard Scheller)

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Membrane and cellular Biophysics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 208920

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.25	4.25	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have continued our research on membrane fusion, the fundamental step in secretion, viral infection, fertilization and neuro-transmission. Sea urchin egg cortical granule exocytosis, an example of calcium triggered membrane fusion, has served as a model system. We have been investigating how certain proteins (NSF et al.) might regulate the exocytosis of the egg's cortical granules. We have established that cytosol is unable to restore fusion to the isolated exocytotic machinery inactivated by NEM. We have found using immunoblot analysis that sea urchin eggs contain NSF in their cytosol but that this protein is absent from an in vitro preparation which undergoes fusion upon the provision of Ca2+. However, a stable and specific interaction between NSF and the isolated exocytotic machinery can occur under the appropriate conditions. These results suggest that the role of NSF might be in the establishment of a fusion-competent arrangement rather than in membrane fusion *per se*: a novel, NSF-independent mechanism of fusion.

The relative roles of inactivation and heterogeneity were evaluated further in the isolated planar cortex from the sea urchin eggs using multiple solution exchange protocols and perfusion at different rates. No evidence was found to support the hypothesis that either rate-dependent or rate-independent inactivation is responsible for the cessation of fusion at calcium concentrations which produced sub-maximal responses in cortical degranulation. Calcium-triggered exocytosis was heterogeneous: exocytotic responses were sub-maximal at certain calcium concentrations because all the granules capable of fusing at those concentrations of calcium had fused. Secretory models featuring calcium-dependent inactivation are inappropriate for describing cortical degranulation in the sea urchin egg. The minimum number of calcium ions involved in the fusion process was estimated by considering the behavior of a single, step-like transition at a threshold calcium concentration. This analysis suggests that more than four calcium ions are involved in triggering exocytosis. Our model, which describes both the kinetic and steady-state features observed in sea urchin cortical granule exocytosis, has been extended with the addition of a third parameter representing the activation time for fusion complexes. Discrepancies between the model and the initial time course of exocytosis have been eliminated with this modification.

Project Description

Objectives:

The goal of this project is to elucidate molecular mechanisms involved in secretion, viral infection, fertilization, and neuro-transmission and assign functional roles to proteins and lipids involved in membrane fusion by studying the temporal dependence and properties of identifiable stages in calcium triggered cortical granule exocytosis.

Our primary biochemical goal has been to learn more about the proteins which are required for membrane fusion and docking. We plan to assign functional roles to proteins involved in membrane docking and fusion by studying their localization, binding partners, reconstituting their function in docking and fusion, link function to kinetic models, and provide a tangible biochemical and physical basis for understanding the mechanism of intracellular membrane fusion.

The specific objectives of the kinetic arm of this project have been to 1) evaluate the relative roles of rate-dependent inactivation and heterogeneity using multiple solution exchange protocols and slow perfusion at different rates of increasing calcium concentrations (calcium ramps), 2) extend a general, kinetic model which relates the rate and extent of triggered exocytosis to the number, distribution and efficacy of activated fusion complexes, to include an activation time for fusion complexes and 3) examine the consequences of heterogeneous thresholds in triggered exocytosis and estimate the minimum number of calcium ions involved in the fusion process.

It is to be hoped that, after identifying the proteins required for various processes during vesicle transport and membrane fusion, we will be able to identify human disorders in which these proteins are involved in pathogenesis and subsequently, develop strategies for therapeutic intervention.

Methods Employed:

Preparation of Cell Surface Complex (CSCs)

Sea urchins were purchased from Marinus (Long Beach, CA) and were maintained in aquaria in artificial sea water (435 mM NaCl, 40 mM $MgCl_2$, 15 mM $MgSO_4$, 11 mM $CaCl_2$, 10 mM KCl, 10 mM Hepes, 1 mM EDTA, pH 8.0) at a temperature of 14oC. Eggs were obtained by injecting 0.5 M KCl into the intracoelomic cavity and the jelly coat was removed by passing through 90 μm nylon mesh. The isolated exocytotic machinery (CSCs) was isolated in the following way. Eggs were washed 3 times and resuspended in IM (IM: 220 mM K-glutamate, 500 mM glycine, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM benzamidine, pH 6.7) at 4°C and were homogenized gently until microscopic examination showed that no intact eggs remained. After homogenization, CSCs were pelleted at 700 x g for 1 minute and resuspended in fresh IM.

Preparation of Egg S100 Cytosol

Eggs were washed into ice cold IM containing 2.5 mM MgATP, 5 mM DTT, 10 μg/ml aprotinin, pepstatin and leupeptin (cytosol buffer). Eggs were resuspended in an equal volume of cytosol buffer and were gently homogenized by 3 strokes of a loose-fitting teflon pestle in a Potter homogenizer. The homogenate was centrifuged for 1 hour at 4°C and 100,000 x g . The supernatant (S100) was retained, passed through a G25 Sephadex column in IM and frozen at -70°C until use.

Measuring Exocytosis

100 μl aliquots of CSCs were dispensed into flat-bottomed microtiter plates. Exocytosis was triggered by the addition of 100 μl of Ca+/IM to give the desired Ca^{2+} concentrations. Ca^{2+} concentrations were determined using a calcium-selective electrode and was measured in a microtiter plate reader as a decrease in absorbance at a wavelength of 405 nm.

NSF-binding Assay

The binding of NSF to egg cortical fragments was performed in the following way. Planar cortices were isolated by attachment to polylysine-coated plastic petri dishes. They were incubated with cytosol under a variety of conditions. After an appropriate incubation time, the cortices were exhaustively washed and protein detached from the solid support by incubation with 0.1 M Tris, pH 11. Protein was precipitated using trichloroacetic acid and dissolved in sample buffer.

Isolation of endoplasmic reticulum membranes

"Crude" endoplasmic reticulum membranes were isolated by differential centrifugation. Highly purified, salt extraction membranes were prepared by isolating rough endoplasmic reticulum fraction by isopycnic centrifugation followed by extracting membranes with 1M NaCl. Extracted membranes were isolated by isopycnic centrifugation and used in experiments.

Fusion assay for endoplasmic reticulum membranes

For fusion to occur, non-labeled membranes and membranes containing R18 (octadecyl rhodamine B) incorporated into the membrane at a fluorescence self-quenching concentration were brought into contact with polyethyleneglycol (PEG). Fusion was triggered by the addition of GTP and detected by an increase in R18 fluorescence.

Electrophoresis and Western Blotting

Samples were subjected to electrophoresis in 12% polyacrylamide gels and transferred to PVDF membranes. Proteins were detected ECL detection system.

Kinetics

The exocytotic response to defined calcium concentrations was inferred from changes in light scattering intensity obtained using a microscope light scattering photometer. Fast perfusion of solutions was achieved using a flow chamber specifically designed for use with multiple solution exchange protocols (Kaplan, Bungay, Sullivan, Zimmerberg, 1996). Calcium ramps were generated using a gradient maker and a peristaltic pump operating at a flow rate of 3.5 ml/min and total volumes of 40 and 80 ml corresponding to linear gradients in the total calcium lasting approximately 11 and 23 minutes. Curve fitting and model development were performed using commercially available software.

Major Findings:

1) One of the paradigms for NSF-dependent membrane fusion is that cytosol is able to restore activity to NEM-inactivated fusion systems. We have shown that cytosol fails to restore fusion to the NEM-inactivated isolated exocytotic machinery, even when inhibition is sub-maximal. There is no concentration of NEM whose inhibitory effect is overcome by the addition of cytosol. It has now been firmly established that cytosol has no effect upon the Ca^{2+}-sensitivity of cortical granule exocytosis in vitro.

2) We have found that sea urchin eggs contain NSF. This protein is located in the cytosol but not in the isolated exocytotic machinery.

3) Sea urchin egg NSF has the same membrane binding properties that have been found for NSF from other sources. In the presence of non-hydrolyzable analogues of ATP, NSF binds to the isolated exocytotic machinery. This binding is prevented by treatment with NEM and recombinant NSF competes for the same binding sites. This binding has no effect on calcium-stimulated exocytosis. These data all suggest that membrane fusion during cortical granule exocytosis is downstream of the actions of NSF.

4) By Western blotting, we have identified proteins that cross-react with polyclonal antisera raised against the mammalian SNARE proteins, syntaxin and synaptobrevin.

5) No evidence for rate-dependent inactivation was observed using calcium ramps: all granules fused and no shifts in the dynamic response to regions of higher calcium concentration were observed. The extent of fusion was independent of the rate of calcium delivery for rates corresponding to an ~ 60 μM change in calcium concentration occurring over 300 msec or eight minutes. The calcium sensitivity of individual granules within a population of exocytotic granules is heterogeneous and heterogeneity does not arise from rate-dependent inactivation.

6) The measured calcium activation curve can be described by a log-normal cumulative distribution function where the first derivative represents the underlying probability density function for fusion thresholds.

7) More than four calcium-dependent events could account for the measured calcium activation curves observed in cortical granule exocytosis.

8) Discrepancies between the initial time course of exocytosis and our two parameter model have been resolved with the addition of a third parameter representing the activation time for fusion complexes.

Significance to Biomedical Research and the Program of the ICD:

Intracellular membrane fusion is crucial to the functioning of all cells. It is required for exocytosis, endocytosis, secretory protein trafficking and neurotransmitter release to name but a few. Understanding the mechanisms behind membrane fusion should allow us to develop techniques to intervene in pathological conditions characterized by dysfunctions in these processes.

The establishment of the origin of sub-maximal exocytotic responses being due to heterogeneity is important because a) differentiation between calcium-dependent inactivation and heterogeneity is required for the development of robust secretory models, b) sub-maximal responses are a feature common to all calcium-triggered exocytotic systems, and c) the goal of relating specific proteins with specific steps in exocytosis can only proceed if the steps are correctly identified. We have refined a theoretical model which can explain the complex biological behavior observed during triggered exocytosis. Human maladies of secretion, such as diabetes, infertility, and neuronal and endocrine dysfunction, may now be tested for lesions in the number, distribution, efficacy and activation of fusion complexes at membrane fusion sites.

Proposed Course:

1) We shall determine the proteins in the sea urchin exocytotic machinery which interact with mammalian NSF using an affinity isolation procedure followed by SDS-PAGE and microsequencing. The data obtained will allow us to develop probes for use in determining the roles of these proteins in vesicular transport and fusion.

2) We shall use monoclonal antibodies raised against thiol-containing proteins in order to identify the fusion-related proteins whose thiols are required for fusion.

3) Antibody production. Antibodies to appropriate peptide sequences will be produced by conventional techniques for use in Western blotting, immunocytochemistry and functional studies. Since functional studies will require large amounts of antibody and since species-specific antibodies are more likely to neutralize biological activity, we will obtain polyclonal antibodies against sea urchin NSF, SNAPs and SNAREs.

4) Localization with immunogold labelling. We will perform localization studies on the SNAP and SNARE proteins by immunoelectron microscopy. It will be very interesting to determine if the SNAREs are localized to the contact zone between the plasma membrane and the cortical granules.

5) Selected sea urchin SNARE proteins will be cloned utilizing information from microsequence data as well as strategies involving immuno-identification of clones in expression libraries. After cloning and sequencing, bacterially-expressed fusion proteins will be added to cortical granule fusion reactions in order to probe function.

6) The probability density function for calcium thresholds will be investigated using high resolution calcium titration of the exocytotic response. If the function is discrete, then the number of different thresholds present may be resolvable. Since the origin of threshold heterogeneity is unknown, a resolvable number of thresholds may be indicative of a family of fusion complexes. The efficacy and activation time of fusion complexes will be evaluated as a function of calcium concentration. The results from local and kinetically specific assays for docking, activation, and fusion including flash photolysis, dynamic light scattering, and polarization will be evaluated using the theoretical model.

Publications:

Kaplan DJ, Bungay P, Sullivan J, Zimmerberg J. A rapid-flow perfusion chamber for high-resolution microscopy. J Microscopy, 1996;181:286-297.

Sokoloff AV, Whalley T, Zimmerberg J. The characterization of N-ethylmaleimide-sensitive thiol groups required for the GTP-dependent fusion of endoplasmic reticulum membranes. Biochem J, 1995;312:23-30.

Vogel SS, Blank PS, Zimmerberg J. Poisson distributed active fusion complexes underlie the control of the rate and extent of exocytosis by calcium. J Cell Biol, 1996;134:329-338.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01416-04 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Interactions in Three-Dimensional Tissue Culture

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Margolis	Visiting Scientist	LTPB, NICHD
Others:	S. Glushakova	Visiting Scientist	LTPB, NICHD
	S. Hatfill	IRTA Fellow	LTPB, NICHD
	A. Sylwester	IRTA Fellow	LTPB, NICHD
	J. Zimmerberg	Chief	LTPB, NICHD

COOPERATING UNITS *(if any)*

NASA (N. Pellis)

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Unit on Intercellular Interactions

INSTITUTE AND LOCATION

NIH, NICHD, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.75	3.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The complex cell-cell interactions in tissue are the basis for all of the aspects of normal and pathological immune responses *in vivo*. These cell interactions are altered outside the context of actual tissue. We continue to develop and benchmark system of histocultures of human lymphoid tissue *in vitro* and use this system to study HIV pathogenesis. We have found that the histocultures of human tonsils we develop preserve both their cytoarchitecture, including a key element, a three-dimensional network of follicular dendritic cells, as well as an immune function. *In vitro* immunization of tonsil histocultures (but not of cells isolted from them) with tetanus toxoid (TT) results in the production of anti-TT IgG. Histocultures of lymphoid tissue are also able to support productive viral infection with two laboratory strains and six primary isolates, of syncytia-inducing (SI) and non syncytia-inducing (NSI) phenotypes, as well as macrophage- and lymphocyte-tropic viruses. Infection with HIV affects both the cellular and the functional aspects of the histocultures in an isolate-dependent manner. Infection with SI HIV-1 isolates resulted in a dramatic decline in CD4+ lymphocytes in histoculture. NSI viruses do not cause such complete depletion of tissue CD4+ lymphocytes. HIV infection greatly affected the immune response of the histocultures to tetanus toxoid. The immune response to TT was dramatically inhibited in histocultures infected with T-tropic HIV isolate. In contrast, infection with M-tropic isolate appears to lead to the enhanced production of antibodies against tetanus toxoid. Mechanisms of HIV-induced dysregulation at various stages of HIV-disease can now be studied in the context of a complex system of cell-cell interactions under controlled conditions *in vitro*.

Project Description:

Objectives:

The Complex cell-cell interactions of tissues form the basis for all aspects of normal and pathological tissue responses in vivo. Cell behavior becomes significantly different outside the context of normal tissue architecture even when many types of cells are co-cultured together in cell monolayers or in cell suspensions.

That is particularly true for the immune system. It is now accepted that the deterioration of this system of cell-cell interactions in the course of HIV infection is the main cause of AIDS. We are continuing to develop and benchmark our sytem of histocultures of human immune tissue in vitro. We have found that these histocultures of human tonsils preserve both their cytoarchitecture and immune function. At present stage of the project we have attempted to check the immune response of this system and how this is modulated by HIV infection. We are also trying to reconstitute the system with exogeneous cells and to image these cells in the tissue.

Methods Employed:

Preparation and culturing of histocultures. Human tonsils or adenoids surgically removed during therapeutic tonsillectomy, were delivered in PBS to our laboratory within eight to nine hours post operation. Lymph nodes were obtained from deceased (not due to AIDS) donors and delivered in culture media on ice within 24 hours post mortem. As evaluated by the trypan blue exclusion test, viability of cells in specimens used in the experiments was approximately 70-80%. Specimens were dissected into 2-3 mm^3 blocks and incubated in RPMI medium 1640 supplemented with 15% FBS and antibiotics, and placed on top of collagen gels at the air-liquid interface, as described elsewhere (33). This media was changed every two to three days and analyzed in a Beckman Glucose II Analyzer to determine he glucose consumption. Ciba Corning 238 Blood Gas Analyzer was used to to determine pH, pCO_2, and pO_2. Alternatively the tissue pieces were placed inside the Rotating Wall Vessel (RWV) filled with culture medium. The RWV is placed on its base and rotated at a speed of 30 - 50 rpm depending on the type and size of tissue blocks. O2 readings are gathered from the blood gas readings performed throughout the culture length. Glucose readings are taken before and after each refeeding of the cultures, so that glucose consumption can be calculated.

HIV infection and virus production. We infected lymphoid histocultures with the laboratory strains of HIV-1, LAV.04 and SF162 and with primary isolates of HIV-1 from infants (USA) #302076, #302144, # 302074, #302056, #302143,#302151. These viruses were obtained through the AIDS Research and Reagent Program, Division of AIDS, NIAID, NIH. To inoculate the lymphoid tissue histocultures, 1-10 :1 of clarified virus-containing medium were applied to the top of each issue block. The multiplicity of infection (MOI) varied from 400 to 900

$TCID_{50}$ (50% tissue culture infective dose) per block. The medium was changed 24 to 48 hours post infection. At various times after infection, samples of each culture were collected for analysis. The concentration of p24 in the medium and inside the tissue was measured by HIV-1 p24 antigen ELISA ("Retro-Tek", Cellular Products Inc., Buffalo, N.Y.). Infectious titers of viruses in the culture medium were quantified by an infectivity assay (25) and by end-point-dilution cultures of PBMC (23). The number of infected and control histocultures analyzed in each experiment was between 10 and 36.

Analysis of the tissue. Immunohistochemistry and *in situ* hybridization. Blocks of histocultures were fixed with 4% paraformaldehyde at different times after the cultures were set up. The fixed tissues were dehydrated, embedded in paraffin, and sectioned for immunohistochemical staining. The top section in each series was stained with hematoxylin-eosin. Subsequent sections were immunostained for B cells, follicular dendritic cells (FDC), and macrophages, and p24 using monoclonal mouse antibodies against, CD20, CD21, CD68, and p24 respectively. For T cells we stained the sections with rabbit anti-human antibodies against CD3. Anti-CD21, anti-p24, and anti-CD3 antibodies were unlabeled and used as primary antibodies for indirect immunostaining. All procedures were performed according to standard DAKO protocols. For staining of p24, CD3, or CD21, the dewaxed and enzymatically treated sections were incubated with primary antibodies for two hours, washed twice, and then incubated for another 60 minutes with secondary antibodies. To check the specificity of staining, control sections were treated with mouse IgG instead of primary specific antibodies or with labeled IgG in a direct immunostaining assay. No immunostain was observed in these control experiments. Also, no staining for p24 was observed in uninfected control blocks of tonsils.

LAV RNA was detected in infected tissue blocks by *in situ* hybridization with an antisense (AS) probe cocktail (Lofstrand, Rockville, MD) that is complementary to gag-pol (*Hind*III-*Kpn*I), env (*Pvu*II-*Hind*III), pol (*Pvu*II-*Pvu*II), pol/vif/VPR/rev/tat (*Kpn*I-*Kpn*I), and gag (*Sac*I-*Bgl*II) generated from subclones of the BH10 plasmid. As a control, a sense (S) riboprobe cocktail was generated from the same plasmids. Antidigoxigenin alkaline phosphatase antibody was added, and the samples were incubated followed by color development with NBT/BCIP substrate. A section of uninfected negative control tissue and of HIV-positive tissue were included as controls with each set of experimental samples.

Flow cytometry. Control and HIV-infected histocultures of human tonsils were mechanically dispersed into single-cell suspensions. To avoid a loss of cells during standard purification procedures (which our experience suggested could change the ratio of lymphocyte subsets), we used for analysis whole suspensions obtained from tissue blocks. Cell suspensions were stained with monoclonal antibodies conjugated with various fluorophores. Antibodies (Becton Dickinson, San Jose, CA) against the following surface antigens were used: CD3, CD19, CD8. The following antibodies against CD4 were used: Leu 3a, V4 (Becton Dickinson) and OKT4 (ORTO Diagnostics, Raritan, N.J.). Antibodies

were conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP). Stained cells were fixed in 1% paraformaldehyde. After fixation, cells were resuspended in DPBS and analyzed with FACSort using CellQuest software (Becton Dickinson). To estimate the absolute number of cells from different subsets per block, we used the Becton Dickinson Trucount assay in combination with triple staining for CD4/CD8/CD3 or for CD3/CD19/CD45. As a standard, a known number of fluorescent beads were added to the cell suspension prior to flow cytometry. The analysis gates for lymphocytes on light scatter dotplots were established using cells from non-cultured tonsils. Multiple determinations for various cell antigens were performed for each experimental and control cell suspension. Compensation adjustments were made for spectral overlap of FITC and PE or of PerCP and PE, whereas no compensation was required for PerCP and FITC. Between 1000 and 5000 gated events were acquired for analysis. The levels of non-specific binding of fluorescent-conjugated antibodies were obtained in control experiments, when identically treated cells were incubated with mouse IgG conjugated with a correspondent fluorophore.

Confocal microscopy. We used a Leica TCS Confocal microscope with an argon-crypton laser (Omnichrome). Specimens of histocultured tonsils were stained with FITC-labelled anti CD21 antiserum. Histocultured tissues were viewed directly, without sectioning, using long-working-distance objectives. We calibrated the fine focus of the microscope according to (Coble et al., 1992). The images were averaged and stored on the hard disk of a computer. 3D reconstruction was perfomed using Leica commercial software and MetaMorph package.

Major Findings:

1. The human tonsil histoculture system has been established as an *in vitro* model for lymphoid tissue. These histocultures preserve their cytoarchitecture, including their important three-dimensional network of follicular-dendritic cells. *In vitro* immunization of human tonsil histocultures but not of isolated cells with TT, results in the production of anti-TT IgG.

2. Histocultures of lymphoid tissue are able to support productive viral infection with two laboratory strains and six primary isolates, of syncytia-inducing (SI) and non syncytia-inducing (NSI) phenotypes, as well as macrophage- and lymphocyte-tropic viruses.

3. HIV infection of lymphoid tissue histocultures result in the isolate-dependent depletion of CD4+ cells, a hallmark of HIV infection. The most dramatic decline in CD4+ lymphocytes in histocultures was evident with the SI HIV-1 isolates. The NSI viruses do not cause such a complete depletion of tissue CD4+ lymphocytes.

4. HIV infection greatly affects the immune response of human tonsillar histocultures to tetanus toxoid. The immune response to TT was dramatically inhibited in histocultures infected with T-tropic HIV isolate. In contrast infection with M-tropic isolate results in enhanced production of antibodies against tetanus toxoid.

Significance to Biomedical Research and the Program of the ICD:

Various dysfunctions of the human immune system occur due the impared interactions of cells in lymphoid tissue. The most notable example of such dysfunction is HIV-disease. The ability to study the mechanisms of various immunopathological states under controlled conditions *in vitro* and within the context of human lymphoid tissue, is important for answering basic questions on immunopathogenesis including the dysregulation of the immune response caused by HIV.

Proposed Course:

1. To continue to benchmark lymphoid tissues *in vitro*:

   a. To study cell kinetics in this system, in particular locomotion inside/the tissue, exchange between various cellular pools, cell replication and apoptosis.

   b. To study the ability of the system to respond by production of specific antibodies and cytotoxic lymphocytes to primary foreign antigens.

2. To study the structural and functional alterations in the tissue infected with HIV.

   a. To study the deterioration of FDC network in HIV-infected histoculturesas function of time and HIV isolate specificity.

   b. To study the mechanisms of dysfunctional immune responce to foreign antigens. To study separately dysfunction of T, B and FDC cells in the course of HIV infection.

   c. To correlate immune dysfunction with structural alterations and to reconstitute the infected histocultures with functional cells.

Publications:

Glushakova SE, Baibakov B, Margolis LB, Zimmerberg J. Infection of human tonsil histocultures: a model for HIV pathogenesis, Nat Med 1995;1:1320-1322.

Galkina SI, Sud'ina GF, Margolis LB. Regulation of intracellular ph by phospholipase a2 and protein kinase c upon neutrophil adhesion to solid substrata, FEBS Letters 1996, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01417-03 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Energetics of the Interaction Between Water, Membranes, and Macromolecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A.Yergey	Head	SMAMS, LTPB, NICHD
Others:	V. Nguyen	IRTA Fellow	SMAMS, LTPB, NICHD
	X. Chen	IRTA Fellow	SMAMS, LTPB, NICHD

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Metabolic Analysis and Mass Spectrometry

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.8	2.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Preliminary determination of the energy minima for collision induced dissociation of water molecule losses from hydronium ion clusters with water are consistent with published values for solvation enthalpies of the proton. 2. Mass spectra showing the presence of stable clusters of water around tetraalkyl ammonium ions have been obtained. Current theories of water structure in the vicinity of ions suggests the possibility of these clusters, but direct evidence for them has not existed previously. 3. The average rate at which labile protons of angiotensin II exchange with water- has been measured by hydrogen/deuterium (H/D) exchange. We found that 12/16 labile protons exchange with water within 0.02 minutes while the remaining four exchange within 4.0 minutes. These measurements, when coupled with controlled fragmentation of the molecule, allowed its solution phase conformation to be characterized. Adding small amounts of lipid to the peptide solution before the H/D exchange showed the peptide's conformation had altered and 4/16 protons did not exchange within 2 hours. 4. Electrospray mass spectra of fresh samples of 1-40 residue beta-amyloid protein (betaA4) ranging from 250 uM to 6 uM show a mixture of monomeric and aggregated forms of the peptide. The aggregates range from dimer to tetramer and are more abundant at higher concentration. The principal monomeric ion has 5 charges resulting from the addition of 5 protons, while the principal dimer has 9 charges. After aging the samples in water baths at 37°C, we observed a decrease of about 0.6 for the average charge associated with the monomer. This is interpreted as a loss in charge site availability and is associated with a conformational change in the monomer to a more compact form. This is the first report of conformational differences in betaA4 by mass spectrometry.

Project Description:

Objectives:

The purpose of this work is the characterization of the energy required for non-covalent bonds to exist between molecules and ions in solution. Knowledge of the energetic requirements of such non-covalent bonds is fundamental to an understanding of molecular interactions and the changes in conformations that are integral to them. The bonds of particular interest for this work are those involved in the interplay of solutes, including lipids, amino acids and small model peptides with water. We are using several techniques of physical mass spectrometry to study the energetics of water and gas phase ions as well as to study the basic interactions that are responsible for conformational changes in peptide molecules.

The objective of the thermodynamic investigations is determining solvation enthalpies of ions of interest in what may be the best possible approximation of the ideal case of isolated ions at infinite dilution. The approximation is effected using gas phase ion molecule chemistry under conditions of either equilibrium or low pressure collisional dissociation. Under these circumstances, ions, the solute species of interest, can be viewed as isolated entities surrounded by selected numbers of water molecules. The objective of the conformational studies is determination of solvent and structural dependent alterations in peptide conformations as measured by the rates at which labile hydrogens are exchanged in solutions of heavy water. In the long term we see these objectives leading to an increased understanding of the fundamental aspects of protein and peptide folding and the basic thermodynamics that underlie the ordering of membranes.

The initial goal of the solvation enthalpy portion of the project is determining the enthalpy of solvation of $(C_6H_5)_4As^+$ and $(C_6H_5)_4B^-$ Independent of any extra-thermodynamic assumptions. At present, these lipophyllic ions are assumed to have identical solvation thermodynamics. While there is good reason to argue that this assumption is questionable, it is unverifiable due to the impossibility of making calorimetric measurements of isolated ions. The assumption of equivalence for these two solvation enthalpies has substantial fundamental implications for the nature of the interaction of water with hydrophobic surfaces such as lipid membranes. The present mass spectrometric investigation of these ions may be able to resolve the question of whether water interacts with lipid membranes through hydrogen bonding or dipole potentials.

Methods Employed:

Hydration Thermodynamics: Small water cluster measurements.

Under equilibrium gas phase ion-molecule reaction conditions, measurements of free energy and enthalpy are comparable to the best values of bulk phase solvation energetics. The importance of this comparability is, however, that the values determined by the gas phase measurements are independent of any extra-thermodynamic assumptions. In other words, the equilibrium gas phase measurements potentially represent a better approximation to the thermodynamic ideal value than do the bulk phase measurements. The experimental challenge is to attain equilibrium thermodynamic conditions under circumstances that give adequate ion inensities. The critical aspect of the work is achieving the equilibrium conditions in the "high pressure" ion formation portion of the mass spectrometer while not perturbing ion intensity distributions in the process of making measurements in the low pressure portion of the instrument. The equilibrium states must be obtained in combination with stable conditions for the formation of the ion clusters of interest. In our case that requires the introduction of the non-volatile organic solutes into the vapor state under well defined conditions of temperature, pressure and solvent concentration. The experimental approach being used is a reduced pressure electrospray ion source in conjunction with a single stage pressure of reduction prior to mass analysis in a cryopumped mass analyzer. This approach has recently been shown by Kebarle and co-workers to yield equilibrium ion cluster distributions and to reproduce alkali ion ΔH_{solv} values obtained under other conditions.

Hydration Thermodynamics: Large water cluster measurements.

Under the kinetically limited conditions of ion formation in the high pressure region of an electrospray ionization apparatus, ions are produced surrounded by excess solvent molecules. In analytical applications of electrospray ionization, this excess water is stripped from the analyte ions by a combination of electrostaticacceleration and thermal energy. If one were to reduce electrostatic accelerations in combination with lowered thermal energy input, then ions could be delivered to the mass analyzer still surrounded by solvent molecules. Using this technique, Castleman and others have shown that the hydronium ion, H_3O^+, can be observed surrounded by between 1 and 50 water molecules. It has also been shown that the relative intensity of the hydronium surrounded by 20 waters is anomalously larger than expected. This anomaly has been associated with the formation of a clathrate-like structure of hydrogen bound water molecules in a regular dodecahedral structure, and the presence of 20 water molecules has been called a magic number distribution.

The energy with which an individual water molecules is bound in such complexes can be measured by carefully controlled physical interactions within the mass spectrometer. Specifically, causing a cluster ion with defined energy and containing some number of solvent molecules, $[M\cdot(H_2O)_n]^+$, to collide with a neutral gas molecule, argon for example, results in a fragmentation process: $[M\cdot(H_2O)_n]^+ + Ar \rightarrow [M\cdot(H_2O)_{n-1}]^+ + H_2O + Ar$. The process is commonly referred to as collision induced dissociation, CID. The minimum energy at which a CID occurs can be determined. This minimum energy is related directly to solvation enthalpy. It is therefore possible to use large, kinetically limited, water cluster ions to determine solvation enthalpy.

This large cluster ion approach is complimentary to the equilibrium studies because it provides access to extensively hydrated ions that are not obtained in the equilibrium studies. In addition, these cluster ions can be used to characterize solute/solvent interactions by the extent to which the magic number cluster ions form.

Protein/Peptide Conformation Studies:

Electrospray ionization can be used to probe conformations of proteins and peptides while being operated in a mode that is very similar to that used anlytically. Labile hydrogen atoms on proteins and peptides are subject to exchange with protons that are present on solvent molecules. The rate at which such exchanges occur are a function of solvent conditions, local molecular structures as well as the conformation of the peptide. These exchanges can be studeid with great specificity by replacing solvent protons with the common hydrogen isotope, deuterium. Each proton lost from the peptide and replaced with a deuteron results in the gain of one atomic mass unit for the peptide. This process of H/D exchange is readily followed mass spectrometrically, and thus the effects of amino acid substitutions and changes in solvent conditions on conformation can be followed as a function of exchange time. Additionaly, some peptide molecules, such as the ß-amyloid protein associated with Alzheimer's Disease that forms aggregates or the collagen-like petides that form stong triple peptide helical structures, will tend to yield electrospray ionization mass spectra that are indicative of their structures in solution.

Major Findings:

1. *Hydration Thermodynamics*: Small water cluster measurements.

Measurement of the enthalpy change for the successive additions of water molecules to a hydrated proton cluster ion are the archetypal reaction for studies of hydration. This reaction has been studied extensively in the past, and is being used as the model and proof of method in the present work. Since the hydrophobic quaternary ions initially of interest can be introduced to the ion source from a flowing water stream, use of the water cluster system as the starting point for measurements is a reasonable approach. We have mass spectra of water clusters of the proton in the equilibrium source for which the electric fields and temperatures within the source are consistent with equilibrium. Ion intensity ratios consistent with equilibrium for well determined concentrations of water vapor will be available in the near future.

2. *Hydration Thermodynamics*: Large water cluster measurements.

a) CID studies of water clusters in the region of the magic number clathrate have been made. Determination of the energy minima for the processes awaits some refinement in data analysis techniques, but preliminary values obtained for the processes are consistent with published values for solvation enthalpies of the proton.

b) The behavior of a number of organic and inorganic ionic species has been examined for their ability to form magic number clathrates with water under electrospray ionization conditions. To date, the only hydrated ions which have been reported to form the stable clathrate cages are hydronium and ammonium, H_3O^+ and NH_4+. We have found that some of the tetraalkyl ammonium ions, R_4N^+, where R varies from methyl through pentyl as well as some of the alkali and alkali earth metal ions form such clathrates. Specifically, the tetraethyl through tetrapentyl ammonium ions, K^+ and Cs^+ all give rise to the 20 member anomaly in the water cluster distribution, while the tetramethyl ammonium, Li^+, Na^+, Mg^{2+}, Ca^{2+} and Ba^{2+} ions do not. Current theories of water structure in the vicinity of ions may serve to account for these observations.

Solvent water is considered to be organized around solute ions by either electrostatic or hydrogen binding forces, or some combination of these. One might expect that ions with high surface charge densities to organize water principally on the basis of electrostatic forces, due either to small radii or large numbers of charges. On the other hand, ions with low charge densities might be expected to organize water by hydrogen binding forces. At first, one might think that the alkali and alkali earth ions would organize water electrostatically while the tetraalkyl ammonium ions would organize it through hydrogen bonding. Evidence from solution thermodynamics shows, however that there are transitions in solvation enthalpies that occur between Na^+ and Cs^+ and similarly between tetrametyl and tetrapropyl ammonium ions with the behavior of the K^+ and tetraethyl ammonium ions being somewhat intermediate. These transitions are ascribed to changes in the nature of water's organization around the ions. We feel that our present results represent direct observation of the such transition behavior, and as such, are confirmatory of some of the current theories of the nature of bulk water structure.

3. *Protein/Peptide Conformation Studies:*

a) Studies of H/D exchange of angiotensin II under solvent flow conditions using electrospray ionization have been used as a model for studies of determining peptide conformation. There are 16 protons on the angiotensin II molecule that may be considered labile enough to exchange with solvent protons. Rates of exchange are expected to be fast for protons that are exposed to the solvent and slower for those that are protected by reason of conformational folding. We have determined that 12 of the 16 labile protons exchange with the solvent within an average of 0.02 minutes while the remaining four exchange on the average within 4.0 min. Controlled fragmentation of the angiotensin II molecule in the ion source following the proton exchange chemistry has allowed the slowly exchanging protons to be identified. Two of them are the amide protons bracketing the proline in the center of the molecule and give rise to a ß-turn in its center. The remaining two are associated with each terminal residue and give rise to a second planar region in the molecule.

In order to investigate the possibility of conformational changes that would be associated with interactions with a lipid, the angiotensin II was mixed with cholic acid, which forms lipid micelles of small aggregation number at low concentration. Proton exchange was investigated by rapidly diluting the peptide/micelle mixture in D_2O and introducing the diluted material directly into the mass spectrometer. While any peptide/micelle complex was destroyed by the ionization process, this study showed a very different exchange pattern than had been seen in the earlier one. We observed that three labile hydrogens, associated with the lipophyllic side chains of the central portion of angiotensin II did not exchange with the solvent in the course of 4 hr. This is consistent with that portion of the peptide being bound or dissolved in a lipid micelle.

b) The mass spectra of ß-amyloid protein (1-40) associated with neuritic placques of Alzheimer's Disease have unambiguously shown a conformational change in the peptide that is related to neurotoxicity. Electrospray mass spectra of fresh samples of synthetic peptide, ßA4, at concentrations ranging from 250 μM to 6μM have shown the presence of a mixture of monmeric and aggregated forms of the peptide. The aggregates range in size from dimer to tetramer and are more abundant at the higher concentrations. The most abundant ion of the monomeric form arises from the additon of 5 protons to the molecule, while the most abundant dimer results from the addition of 9 protons. As the original fresh samples are aged by their containers being placed in water baths at 37°C, we observe a decrease of about 0.6 for the average charges associated with the monomer. This is interpreted as a loss in charge site availability and is associated with a conformational change in the monomer to a more compact form. At the same time, the aged samples show no loss in average charges associated with the aggregate forms, but exhibit a loss in their intensity relative to the monomers. We interpret these observations as being the consequence of a relatively "open" conformation of the ßA4 being present in fresh solution. This open form is capable of forming aggregates in a dynamic equlibrium which leads to reduced aggregation upon dilution. Upon aging, the "open" monomeric form alters to a more compact form with a loss in average charge and a reduction in the abundance of aggregates of the "open" form.

This interpretation is consitent with published circular dichroism evidence which shows a random coil form of ßA4 shifing to a ß-sheet form with aging.

Significance to Biomedical Research and the Program of the ICD:

The work undertaken in this project represents studies that are fundamental to issues in physical aspects of structural biology: how much energy is required for reactions to proceed in the aqueous medium and how do peptide conformations change as a function of reaction conditions. As such, these question have the possibility of great generality and significance.

Specifically: 1) We appear to have found ion/water systems that are reasonsble models for assessing the nature of the structure of water in the vicinity of an ion. We have the ability to measure the energetics of these associations. Such models and measurements are fundamentally important to an understanding of basic biological processes. 2) The studies of H/D exchange are the first to report exchange rates for each labile hydrogen using mass spectrometry. In addition, there are no published data that report alterations of a peptide conformation for a chemical interaction other than changing solvent. This study is a potential model system for describing detailed conformational changes of peptide membrane interactions.

3) The results of the mass spectrometry of ßA4 peptide are the first to show any differences between toxic and non-toxic forms of the peptide. That is potentially significant for fundamental studies in vitro of this material that are directed to investigations of the basis for the conformational change. These results are also significant because they show the subtlety of the structural effects that can be probed by electrospray ionization mass spectrometry.

Proposed Course:

The course proposed for each segment of the report can be summarized as one of consolidation of the preliminary results. In the case of the small cluster equilibrium measurements, the course proposed is to complete the initial studies of the proton and alkali earth water cluster thermodynamic measurements and extend the results to the hydrophobic ions tetraphyenyl arsonium and tetraphenyl boride. In the case of the large cluster kinetic studies, the course is to refine data analysis techniques and determine solvation enthalpy values for the alkali and tetraalkyl ammonium ions. Extension of these measurements to the tetraphyenyl arsonium and tetraphenyl boride ions, if possible, might give further insights into the structuring of water around very hydrophobic materials. Finally, in the case of the conformation studies, we propose to concentrate our efforts on the characterization of collagen like peptides. Initially, the question raised is whether or not the electrospray spectra of these materials incorporates water as a necessity as the x-ray crystallographic data of these materials might suggest. A second question is the rate at which waters associated with the collagen-like molecules exchange with the solvent system.

Publications:

Xu Y, Zhang X, Yergey AL. Studies of Transition Metal Ion Complexes with Amino Acids and Peptides by Electrospray Ionization Mass Spectrometry. J Am Soc Mass Spectrom, 1996;7:25-29.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01418-02 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Mitosis at the Cellular Level

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	F. Suprynowicz	Senior Staff Fellow	LTPB, NICHD
Others:	T. Whalley	Visiting Fellow	LTPB, NICHD
	A. Sokoloff	Visiting Associate	LTPB, NICHD

COOPERATING UNITS *(if any)*

Laboratory of Biochemistry, NHLBI (S.-K. Kong, Ph.D.); Dept. Of Physiological Sciences, Univ. of Newcastle, U.K. (L. Groigno, Ph.D.)

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Membrane and Cellular Biophysics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been discontinued.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01419-02 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Localization of Membrane Fusion Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Zimmerberg	Chief	LTPB, NICHD
Others:	T. Whalley	Visiting Fellow	LTPB, NICHD
	A. Sokoloff	Visiting Associate	LTPB, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Membrane and Cellular Biophysics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been discontinued.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01420-01 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Calcium Triggered Membrane Trafficking in Sea Urchin Eggs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Vogel	Senior Staff Fellow	LTPB, NICHD
Others:	M.S. Cho	Biologist	LTPB, NICHD
	M. Terasaki	Senior Staff Fellow	LN, NINDS
	T. Whalley	Visiting Fellow	LTPB, NICHD
	S. Beushausen	Visiting Scientist	LN, NINDS
	D. Lester	Staff Scientist	FDA

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Section on Membrane and Cellular Biophysics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been discontinued.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 01501-05 LTPB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Involvement of Lipids in Biological Fusion Intermediates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Chernomordik	Visiting Scientist	Head, ULMF, LTPB
Others:	A. Chanturiya	Visiting Associate	LTPB, NICHD
	E. Leikina	Visiting Associate	LTPB, NICHD
	I. Markovic	IRTA Fellow	LTPB, NICHD
	A. Bailey	Visiting Fellow	LTPB, NICHD
	J. Zimmerberg	Chief	LTPB, NICHD

LAB/BRANCH

Laboratory of Theoretical and Physical Biology

SECTION

Unit on Lipids in Membrane Fusion

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.25	4.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

While the specificity and timing of membrane fusion in diverse physiological reactions, including virus-cell fusion, is determined by proteins, fusion always involves the merger of membrane lipid bilayers. We have isolated a lipid-dependent stage of cell-cell fusion mediated by influenza hemagglutinin, HA, and triggered by cell exposure to mildly acidic pH. This stage precedes actual membrane merger and fusion pore formation; but is subsequent to a low pH-induced change in HA conformation including insertion of HA fusion peptide into the target membrane. The low pH conformation of HA is required not only to achieve this lipid-dependent stage but also, downstream of it, to drive fusion to completion. The lower the pH that is applied to trigger fusion and, thus, the more HA molecules are activated, the less profound is the dependence of fusion on lipids. Membrane-incorporated lipids affect fusion in a manner that correlate with their dynamic molecular shape, a characteristic which determines a lipid monolayer's propensity to bend in different directions. To further elucidate the pathway and the mechanisms of membrane fusion in a relatively simple experimental system we also studied fusion of phospholipid vesicles to planar phospholipid bilayer membranes. Surprisingly the fusion intermediates detected in this protein-free system (hemifusion and flickering fusion pores) appear to be very similar with the known structural intermediates of biological fusion. These data, and the lipid sensitivity of HA-mediated fusion, are consistent with the stalk hypothesis of fusion suggesting that fusion proteins begin membrane merger by promoting the formation of a bent, lipid-involving, stalk-like intermediate common for biological fusion and the fusion of purely lipidic bilayers.

Project Description:

Objectives:

Biological fusion is thought to be mediated by specialized fusion proteins, and is a multistep reaction in itself. Our goal has been to determine the fusion pathway as the sequence of the structural lipid-involving intermediates and to uncover the physical forces that drive rearrangement of two membrane lipid bilayers into one after activation of fusion protein. Through what structural intermediates proceeds this drastic rearrangement of two lipid bilayers into one? Our working hypothesis is that the different membrane fusion systems, including fusion of purely lipid bilayers, involve a consecutive formation of two classes of transient and highly bent intermediates: stalks -- lipidic connections between contacting monolayers of fusing membranes, and pores. The stalk-pore hypothesis suggests that the energetics of hemifusion and fusion pore intermediates depends on the spontaneous curvature of different membrane monolayers. We tested this prediction by specifically altering the lipid composition of different monolayers of biological membranes in influenza HA-mediated fusion. To further investigate the lipid rearrangements in fusion and the role of fusion proteins we also studied the fusion of phospholipid vesicles to planar phospholipid bilayer membranes with a set of assays which will allow us to distinguish a hemifusion intermediate from fusion pore formation, a fusion event from vesicle lysis, and flickering of a fusion pore from its irreversible expansion.

Methods Employed:

Cell-cell fusion.

We studied low pH-triggered fusion of HA-expressing cells with prebound human red blood cells, RBCs. RBCs were labeled with fluorescent membrane dye R18 or with fluorescent water-soluble dye, carboxyfluorescein. To compare the lipid sensitivity of cell-cell fusion mediated by different strains of HA we will use HAb2 cells (A/Japan/305/57 strain of influenza virus HA ([Doxsey, 1985 #197]); CHO-K1 cells expressing the X:31 strain of influenza virus HA (referred to as HA300a cells) or expressing the GPI-anchored X:31 HA (BHA-PI cells) ([Kemble, 1993 #213; Kemble, 1994 #214]). Cells were pretreated by 5 mg/ml trypsin in the presence of 250 mg/ml neuraminidase. Cells were washed twice by phosphate-buffered solution (PBS) and then incubated for 10 min with a 1 ml suspension of RBCs or RBC ghosts (0.05 % hematocrit) at room temperature (20-22°C). The unbound RBC were removed by 3 washings with PBS. HA-expressing cells with bound RBCs (~ 0-2 erythrocytes per cell) were then used for experiments. In some experiments we evaluated the effects of exogenous lipids and some enzymatic treatments on the RBC binding to cells. We randomly selected several areas of the dish and screened at least 200 cells to find the average number of RBCs bound to one HA-expressing cell.

All fusion experiments were performed at room temperature (20-22°C). Fusion was triggered by replacing PBS with an isoosmotic buffer titrated by citrate to acidic pH (pH 5.0, if not stated otherwise). After incubation of cells at low pH for 2 minutes (if not stated otherwise), the acidic solution was replaced by PBS. The extents of fusion, as defined by dye redistribution from RBCs (or RBC ghosts) to HA-expressing cells, were quantified using fluorescence microscopy as the ratio of dye-redistributed bound RBCs to the total number of the bound RBCs.

Vesicle-planar bilayer experiments.

Planar bilayers were formed by Montal-Mueller technique across a ~200 mm aperture in Teflon partition separating two aqueous compartments filled with the buffer A: 400 mM KCl, 20 mM $CaCl_2$, 1 mM EDTA, 10 mM HEPES, pH 7.5 (770 mosm) or with buffer A supplemented with 20 mM stachiose (780 mosm) in some experiments with osmotically balanced vesicles. The chamber, milled from perplex, was similar to that described by Niles and Cohen (1988). The chamber was placed between an objective and condenser lens mounted on a microbench system (Spindler and Hoyer, Germany). A 10x, 0.21 N.A. lens was used for observation of planar bilayer under bright field illumination and a 40x, 0.5 N.A. lens (Nikon) was used as an objective for fluorescence. Fluorescent images are obtained with two sets of interference filters (Omega Optical, Brattleboro, VM): 545 nm excitation and 590 nm emission filters for rhodamine fluorescence; and 485 nm excitation and 520 nm emission for calcein fluorescence. Images are projected onto a SIT camera (Hamamatsu) connected to a VCR, videoprocessor, monitor and computer.

We have used giant unilamellar vesicles (~10 mm diameter) loaded with 200 mM calcein, 5 mM n-propyl gallate, 10 mM MES, pH 6.7 (835 mosm) for experiments with osmotically stressed vesicles or with the same solution diluted 10 times with the buffer A to 780 mosm for experiments with osmotically balanced vesicles. 10 mg/ml of channel-forming antibiotic nystatin was added to these solutions. Vesicles were formed from the mixture of asolectin, ergosterol and R-PE (70:20:10, w/w %) by the Kim and Marty technique in the modification described in [Niles and Cohen, 1987]. Lipid vesicles were placed on planar bilayer by manipulation of a glass micropipette containing suspension of vesicles. Stirring of the *cis* compartment removes unbound vesicles. Two sided nystatin channels were formed in the vesicles but not in the planar membrane by adding 50 mg/ml of nystatin to the *cis* compartment. Thus vesicle membranes were labelled by fluorescent lipid in self-quenching concentration, and by ionic channels such as nystatin or porin. The aqueous volume of vesicles contained fluorescent water-soluble probes. In addition, the giant size of the vesicles used in these experiments allowed us to study vesicle-planar bilayer fusion using admittance measurements with no nystatin added.

Major Findings:

We found a lipid-sensitive stage common to fusion mediated by HA of different strains of influenza (Japan/305/57 and X-31 strains) and for hemifusion mediated by GPI-anchored HA. The effects of the different lipids correlated with their dynamic molecular shapes The lipid-sensitive stage, arrested by inverted-cone-shaped lysophosphatidylcholine (LPC) and promoted by cone-shaped oleic acid, preceded or coincided with lipid mixing as assayed by membrane dye redistribution from labelled to unlabelled cells upon their fusion. This stage was also upstream of fusion pore formation assayed both as aqueous dye transfer between cells, and by electrophysiological recording. The lipid-sensitive stage is subsequent to the low pH-induced conformational changes in HA upon its activation. The insensitivity of the LPC-arrested stage to neuraminidase, cleaving the sialic acids required for HA1- receptor binding, suggests that LPC blocks fusion at a "committed state" where the fusion peptides of HA are already inserted into the target membrane. Thus, when fusion is blocked by adding inverted-cone-shaped lipids fusion proteins still change their conformation. They can remain in this triggered conformation until the lipid block is removed by either removing added lipid or adding lipid of the opposite molecular shape. Fusion ensues, even if the triggering conditions are no longer present. Changing lipid composition is enough to release the block so that fusion goes to completion. It is as if fusion protein conformational change can do a finite amount of work, and lead to a finite reduction in the energy barrier for fusion, and lipid composition sets that energy barrier.

To clarify the fusion pathway for purely lipidic bilayers, fusion of giant unilamellar liposomes (5-15 mm in diameter) with planar lipid membranes was studied by combination of four independent assays which allowed us to distinguish a hemifusion intermediate from fusion pore formation, a fusion event from vesicle lysis, and flickering of a fusion pore from its irreversible expansion. Hemifusion was seen as membrane dye redistribution and dequenching without any release of water-soluble dye or conductance changes. Fusion pore opening was seen as a release of water-soluble dye accompanied by fast and significant increase of the measured conductance, when the high resistance of the planar bilayer was shunted by the ionic pathway via the high conductance of the permeabilized vesicle membrane, connected to the planar bilayer by the conductance of a fusion pore. The conductance measurements allowed us to distinguish fusion of vesicle to planar bilayer from vesicle lysis, and, in addition, to follow the flickering of the fusion pores. Time-resolved records of pores were surprisingly similar to pores described for viral or exocytotic fusion. This flickering fusion pore formation was often preceded by membrane hemifusion and followed by complete fusion of liposome with planar bilayer observed only for vesicles under osmotic tension. Thus, vesicle/planar bilayer fusion involves hemifusion and formation of transient fusion pores, both stages described for protein mediated biological fusion.

Significance to Biomedical Research and the Program of the ICD:

Although the specific protein-lipid interactions in fusion remain to be understood, the similarity between the effects of membrane lipid composition on HA-mediated fusion and those reported earlier for low pH-, Ca^{2+}-, and GTP-dependent fusion and for fusion of purely lipidic bilayers suggest that proteins mediate disparate fusion processes by altering elastic energies of biological membranes to facilitate bending of membrane lipids into stalk-like intermediates. Knowing how incorporation of different lipids into fusing membranes affect different fusion stages will help to further determine the fusion pathway as the sequence of the structural lipid-involving intermediates and to uncover the physical forces that drive rearrangement of two membrane lipid bilayers into one. The similarity of the characteristics of fusion pores, kinetics of their formation and expansion in lipid bilayer fusion with the reported characteristics of fusion pores in protein-mediated fusion allow us to draw important conclusions on the role of proteins in biological fusion. The existence of very small flickering fusion pores in exocytosis has been considered in the literature as an important evidence of the model of a proteinaceous fusion pore similar to gap junction channels. Our experiments remove this argument. Identification of the pathway of lipid vesicle fusion to the planar bilayer and the parameters of the system, which control this reaction, will bring us closer to understanding the mechanisms of lipid bilayer rearrangements during fusion.

Proposed Course:

1. To evaluate the role of different membrane monolayers in biological fusion. The stalk-pore hypothesis suggests that the energetics of hemifusion and fusion pore intermediates depends on the spontaneous curvature of different membrane monolayers. We will continue to test this prediction by specifically altering the lipid composition of different monolayers of biological membranes.

2. To dissect cell-cell fusion mediated by influenza HA and baculovirus gp64 into distinct stages by applying lysolipids or low temperature. The reversibly arrested fusion stages will be identified and characterized by fluorescence and electron microscopy. We will use cross-linking techniques to stabilize, isolate and characterize the multimolecular complexes, containing viral fusion proteins, and appearing only upon their activation by low pH.

3. To systematically study the effects of different factors on lipid bilayer hemifusion, fusion pore formation and evolution.

Publications:

Chanturiya AN. A comparative study of planar lipid membranes formed by Montal-Mueller and Mueller-Rudin techniques. Membr Cell Biol, 1996;13:235-240.

Chanturiya AN. Filter switching device for dual wavelength videoimaging. J Fluorescence, 1996, in press.

Chanturiya AN, Nikolaenko AN, Shatursky OYo, Lishko VK. Probing the structure-function relationship of α-latrotoxin formed channels with antibodies and pronase. Toxicon, 1996, in press.

Chernomordik LV. Non-bilayer lipids and biological fusion intermediates. Chem and Physics of Lipids, 1996, in press.

Chernomordik L, Chanturiya A, Green J, Zimmerberg J. The Hemifusion intermediate and its conversion 5to complete fusion:regulation by membrane composition. Biophys J, 1995;69:922-929.

Chernomordik LV, Zimmerberg J. Bending membranes to the task: structural intermediates in bilayer fusion. Curr Opin Struct Biol, 1995;5:541-547.

LCMN

LABORATORY OF CELLULAR AND MOLECULAR NEUROPHYSIOLOGY

Mark L. Mayer, Ph.D., Chief

The Laboratory of Cellular and Molecular Neurophysiology (LCMN) studies receptors and ion channels in the mammalian central nervous system. Investigators in LCMN use the techniques of neurophysiology and biophysics, molecular biology and calcium imaging to analyse a wide variety of brain mechanisms such as the properties of individual receptors and channels, mechanisms underlying synaptic plasticity, and the regulation of gene expression in neurons and glia. Collaborative projects which combine the skills of individual investigators, and the overlapping interests of staff in the laboratory, facilitate a collaborative atmosphere. Regular meetings and seminars allow postdoctoral fellows to become familiar with work over an unusually broad range of topics.

GLUTAMATE RECEPTOR PHYSIOLOGY

Excitatory synaptic transmission throughout the brain is mediated by activation of a complex family of glutamate receptors assembled from subunits encoded by the AMPA, kainate and NMDA receptor gene families. The section of neurophysiology and biophysics under the direction of ***Mark Mayer*** investigates the functional properties of glutamate receptor ion channels. Recent studies in LCMN established that cyclothiazide, an allosteric modulator of glutamate receptor desensitization, acts with complete selectivity at AMPA versus kainate and NMDA receptors, and that alternative splicing of flip/flop domains regulates AMPA receptor allosteric modulation. Sequence alignments for each of the four AMPA receptor subunits and subsequent functional analysis of selected mutants revealed a single amino acid exchange (flip Ser750; flop Asn 750) to underlie regulation of allosteric modulation by alternative splicing. Introduction of the Gln residue present in cyclothiazide insensitive kainate receptors abolished AMPA receptor sensitivity to cyclothiazide.

Mutational analysis of AMPA receptor modulation by cyclothiazide. The exquisite sensitivity of AMPA receptor allosteric modulation to conservative amino acid substitutions (Ser, Asn, Gln) prompted Kathy Partin and Mark Fleck to examine allosteric modulation in a broader range of AMPA receptor GluRA$_{\mathrm{flip}}$ position 750 mutants. Thr, Leu, Tyr and Val did not generate functional receptors; Cys and Met expressed poorly; Asp gave responses only in the presence of cyclothiazide; Ser (wild type) and the remaining mutants Asn, Ala, Gly, Gln, generated robust whole-cell responses to glutamate both in the presence and absence of cyclothiazide, but with kinetics which differed from wild type. The only receptor for which desensitization was essentially blocked by cyclothiazide was wild type GluRAflip. For the remaining functional mutants the rate of onset of desensitization was slowed most for S750A; an intermediate response similar to that for wild type GluRAflop was observed for S750G and S750N; for S750M, S750C and S750Q the rate of onset of desensitization in the presence of cyclothiazide was rapid and not significantly different from control. S750D was unusual in that it expressed no current under control conditions, but large currents in the presence of cyclothiazide.

Aniracetam acts preferentially at flop splice variants. Benzothiadiazides structurally related to cyclothiazide and which produce AMPA receptor allosteric modulation presumably act via common mechanisms. Aniracetam, another AMPA modulator which slows desensitization, has no structural homology to cyclothiazide and thus might act at another site. To determine the influence of position 750 on modulation by aniracetam the same series of mutants was assayed. Aniracetam slowed the onset of desensitization for S750N to a greater extent than for wild type GluRAflip. However, the extent of modulation for S750N was similar to that for wild type GluRAflop suggesting that an Asn residue at position 750 underlies the higher sensitivity of flop splice variants to aniracetam, while for cyclothiazide effective modulation requires a Ser residue.

Deactivation produced by brief pulses of glutamate. Cyclothiazide and aniracetam both slow deactivation, the closing of channels which occurs when agonists are applied for too brief a period to produce desensitization. To determine whether allosteric regulation of deactivation is sensitive to amino acid substitutions which control desensitization responses to 1 ms applications of glutamate were recorded in membrane patches. Cyclothiazide slowed the rate of deactivation of GluRAflip 2-fold, but did not modulate deactivation of GluRAflop. Surprisingly, in view of its weaker effects on desensitization, aniracetam was more effective than cyclothiazide at modulating deactivation, and produced a 4-fold slowing for GluRAflop, with a 2-fold slowing for GluRAflip. Modulation of deactivation by cyclothiazide for S750N, S750A and S750Q followed the same trend as for modulation of desensitization in that clearly the SER residue permits the most efficacious slowing of deactivation . In contrast aniracetam slowed deactivation for S750N and GluRAflop to a greater extent than for the other mutants.

Kinetic modeling of allosteric modulation by aniracetam and cyclothiazide. A 12-state cyclic model for ligand-gated ion channels, with binding of 2 molecules of agonist leading to conformational transitions to a single open state or to agonist bound desensitized states, was used to simulate allosteric modulation of AMPA receptor gating. The model allows desensitization to occur only from closed states. Modulation by aniracetam similar to that observed for GluRAflop was achieved by slowing the closing rate constant 6-fold. As would be expected from a cyclic model, slowing the closing rate constant changes accessibility to ligand bound desensitized states, which results in a slowing of the rate of onset of desensitization. The model predicts a linear relationship between modulation of deactivation and modulation of desensitization by aniracetam, a correlation confirmed experimentally. A kinetic scheme reproducing modulation by cyclothiazide for GluRAflip was achieved through "stabilization" of the agonist bound non-desensitized closed state immediately preceding the open state; this was achieved by slowing the rate of onset of desensitization 10,000-fold and by increasing agonist affinity for the RMA2 state 50-fold. Modulation of the onset of desensitization alone cannot account for the slowing of deactivation, which requires stabilization of the RMA2 state; to achieve slowing of deactivation it was not necessary to slow the closing rate constant.

Permeation of glutamate receptor ion channels by cytoplasmic polyamines. A characteristic feature of ion permeation in selected subtypes of AMPA and kainate receptors is a pronounced biphasic rectification which results from ion channel block by cytoplasmic polyamines. At moderately positive membrane potentials polyamines enter and block the ion channel pore; with stronger depolarization the current increases again, most probably because polyamines themselves permeate through the channel. Mayer and his postdoctoral fellows Robert Bähring and Derek Bowie have now obtained direct evidence in support of this from analysis for the GluR6 subtype of kainate receptor of the blocking action of spermine and two polyamine amide toxins: philanthotoxin 3,4,3 (PhTX) and 4-OH-phenyl-propanoyl-spermine (PPS). Both of drugs these contain spermine with one terminal amine linked via a peptide bond to a tyrosine residue; PhTX contains in addition an amide linked butyryl side chain which extends the width of the molecule along the face of the phenyl ring in the tyrosine residue. When added to the internal solution, depolarization from +50 to +100 mV produces a large increase in outward current for spermine but not for PhTX. For PPS there is 2-fold increase in conductance on depolarization from +50 to +100 mV for PPS. CPK models of PPS and PhTX, when viewed along their most narrow axis, have widths of approximately 7Å (PPS) and 7.5 Å (PhTX). Their low permeability compared to spermine is in excellent agreement with a recent pore size estimate of 7.5 Å made by Burnashev and Sakmann (Heidelberg).

Complex block of glutamate receptors by extracellular polyamines. Analysis of the blocking action of extracellular spermine and philanthotoxin gave responses not predicted by the results obtained for internal block. Spermine block occurred at a low affinity site and showed weak voltage dependence. Equilibrium dose response analysis revealed that the Kd behaved as a U-shaped function of voltage, with a minimum around zero mV, consistent with permeation of spermine as the membrane potential is hyperpolarized. In contrast, PhTX proved to be a potent blocker of GluR6 responses with an equilibrium dissociation constant at -80 mV of 57 nM. However, analysis of equilibrium dose response curves for PhTX block at different membrane potentials failed to reveal a strong increase in affinity with hyperpolarization over the range -20 to -80 mV, even though the affinity decreased 160-fold on depolarization from -20 to +40 mV. Kinetic analysis of responses to PhTX revealed an increase in both rate of onset and recovery from block with hyperpolarization. Such results suggest that externally applied PhTX must also be able to pass through the pore on membrane potential hyperpolarization, but at rates much slower than those observed for spermine, presumably reflecting steric hindrance of the tyrosyl-butyryl substituted amine group in the most narrow part of the channel.

Glutamate Receptor Expression in Glial Progenitor Cells. The functional implications of GluR diversity in the brain are beginning to be elucidated, but the molecular mechanisms that control GluR expression are not yet understood. Experiments in Vittorio Gallo's laboratory established that the growth factors PDGF and bFGF selectively potentiate the expression of GluRA mRNA expression in O-2A glial progenitor cells. Nuclear run-on assays further demonstrated that PDGF + bFGF selectively increased the rate of GluR1 gene transcription, and western-blot analysis showed that GluR1 protein levels were increased 6-fold over control by PDGF+bFGF. Utilizing the whole-cell recording configuration, with agonist applied by fast perfusion, Mark Fleck compared the magnitude of AMPA-evoked currents in O-2A cells grown in low serum medium with those of O-2A progenitor cells grown in the same medium containing PDGF and bFGF. AMPA receptor responses were significantly larger in cells treated with PDGF+bFGF compared with untreated cells grown in low serum medium. Although in both culture conditions

AMPA receptor current amplitude and cell capacitance varied between cells, the slope of the relationship between cell capacitance and AMPA -evoked current amplitude was much steeper for cells grown with PDGF+bFGF (0.54 pA/pF) than for untreated cells (0.08 pA/pF). Analysis of the range of steady-state current densities, derived by normalization to whole-cell capacitance, also revealed two dissimilar distributions with a mean current density approximately 4-fold higher in cells treated with growth factors compared with untreated cells.

Effects of growth factors on AMPA receptor subunit composition. Molecular analysis by Vittorio Gallo's group of mRNA and protein levels in glial progenitor cells revealed the growth factors PDGF+bFGF to produce a selective increase in expression of the AMPA receptor GluRA subunit which when expressed in the absence of the GluRB subunit produces channels subject to voltage dependent channel block by polyamines. To determine whether PDGF+bFGF produced functional expression of new AMPA receptors Fleck and Mayer examined the current-voltage relationship of AMPA receptor responses in PDGF+bFGF-treated and untreated glial cells. This revealed pronounced rectification for PDGF+bFGF-treated but not untreated cells. The proportion of the AMPA activated conductance sensitive to voltage-dependent block by polyamines averaged 0.56 in PDGF+bFGF-treated cells, while in untreated cells the ratio was 0.27. In the brain the properties of GluRs are ultimately determined by the types of subunits expressed in the cell membrane to form functional ligand-gated channels. These experiments reveal that transcriptional regulation of AMPA receptor expression can selectively regulate the functional properties of native AMPA receptors.

PHYSIOLOGY OF HIPPOCAMPAL INTERNEURONS.

GABA containing inhibitory neurons represent only 10% of the total hippocampal neuron population, yet their inhibitory synaptic tone far outweighs synaptic excitation onto pyramidal neurons. Despite the wealth of information known about inhibitory synaptic events received by pyramidal neurons, little is known, beyond the basic physiology, of the varied cell types from which inhibition arises. A major effort in the Unit on Cellular and Synaptic Neurophysiology headed by ***Chris McBain*** is to understand the ionic mechanisms which regulate the activity of inhibitory neurons and how these mechanisms impact hippocampal function under both physiological and pathological conditions. In addition, the group is also studying the participation of inhibitory neurons in the plastic phenomenon of long term potentiation or depression and how changes in the synaptic input to these cells impact the hippocampal network.

Lack of Long Term Potentiation in Hippocampal Inhibitory Interneurons. The hypothesis that excitatory synapses onto hippocampal inhibitory interneurons undergo forms of synaptic plasticity similar to those observed in principal pyramidal neurons has received much attention in the last few years. No general consensus exists however, concerning the presence (or absence) of long term potentiation and depression in non-pyramidal neurons. A large source of this disagreement stems from the experimental paradigms chosen to elicit synaptic plasticity in the various studies, since the vast majority of induction protocols previously used have not permitted the separation of plasticity occurring on principal cells, which then propagates to the inhibitory neuron, from direct plasticity on the interneurons themselves. McBain's group therefore devised stimulation paradigms which allowed characterization of both cellular and passively propagated forms of plasticity occurring on a variety of interneuron subpopulations. Interneurons of the st. oriens/alveus received only a passively propagating form of synaptic potentiation, via the recurrent collaterals of CA1 pyramidal cells, but did not posses a direct cellular form of plasticity. Consequently, even in the absence of a direct interneuron plasticity, these cells were influenced indirectly by protocols eliciting synaptic plasticity in CA1 pyramidal cells. This results in the activity occurring at synapses onto the pyramidal neuron population being mirrored in the activity onto the single interneuron. In st. radiatum, two distinct classes of cells were observed: st. radiatum interneurons which showed neither direct nor propagated forms of synaptic plasticity, and "giant-cells" whose EPSPs were robustly potentiated following a pairing protocol. This potentiation is similar to the LTP described in pyramidal cells and its induction requires NMDA receptor activation. Thus, a large heterogeneity of synaptic plasticity exists in morphologically distinct neurons and suggests that complex changes in the CA1 network properties will occur following the induction of LTP.

Identification of the stratum radiatum giant cells. Prompted by the unique plastic properties of the st. radiatum "giant-cell" type, Katalin Toth investigated these cells further to determine whether they might be interneurons or displaced pyramidal neurons. These cells typically possessed a large, triangular shaped cell body with its apex directed towards st. pyramidale. The dendritic tree was extremely elaborate, branching immediately away from the cell body in two main dendritic shafts, which could extend to, and cross, the hippocampal fissure. In contrast only a relatively restricted dendritic tree emerged from the apical end of the soma. Whole cell recordings made from these neurones revealed cells with a stable resting membrane potential, which displayed little spontaneous action potential activity. Injection of a positive current pulse elicited trains of action potentials which showed marked accommodation. With negative current pulses, a voltage sag was observed, consistent with the presence of the anomalous rectifier current (Ih). Action potential durations were quite broad and were usually followed by an afterdepolarization-hyperpolarization sequence. All these properties more closely resemble principal cells than the properties usually demonstrated by inhibitory interneurons. To determine whether "giant-cells" are truly a new class of inhibitory interneuron or alternatively are excitatory pyramidal cells located in st. radiatum, electron microscopic analysis of the synaptic contacts made by this neuron was required. In cells where the pairing protocol had successfully induced long-term potentiation, subsequent electron microscopy revealed that giant cells made asymmetric synapses onto their target cells, indicating that these cells are excitatory, even though the location of their cell body is atypical. Likewise, synapses made onto the giant cell occurred on specialized dendritic spines which had evaded our detection at the light microscopic level. These data strongly suggest that the giant cell is indeed a principal neuron type whose cell body is placed out of st. pyramidale. Since the giant cell of st. radiatum was the only cell type in which robust LTP was observed, was in fact an excitatory pyramidal neuron, this allowed us to conclude that inhibitory interneurons of the mammalian hippocampus do not possess an NMDA-dependent form of long term potentiation.

Hyperpolarization activated current and its role as a pacemaker. Despite the fact that interneurons constitute only ~10% of the total hippocampal cell population, their role in the regulation of excitability is believed to be crucial. The intrinsic electrophysiological properties of these cells play an important role in the generation of their spontaneous activity.

In fact interneurons of the st oriens-alveus invariably display regular action potential activity even in the absence of synaptic drive. The hyperpolarization-activated current (Ih) has been shown to be involved in the regulation of the firing pattern of thalamic neurones, is present in many different neuronal preparations, and was firstly described as the current responsible for spontaneous cardiac pacemaking. McBain's group therefore determined whether the Ih current was responsible for pacemaking activity in this class of interneuron. Following the isolation and characterization of the basic properties of the Ih current in interneurons, it was determined whether Ih contributed to the pacemaking of the rhythmical firing of interneurons, isolated from their synaptic inputs. Selective antagonists of the Ih current, attenuated the spontaneous firing frequency, while minimally affecting other properties of the action potential waveform. When individual spikes were examined on a slower time scale it was apparent that the attenuation of the firing frequency resulted from a prolongation of the interspike interval by decreasing the slope of the depolarizing ramp leading to subsequent action potential threshold. β-Adrenergic agonists increase spontaneous firing by blocking Ih under voltage clamp conditions. Both norepinephrine (NE) and isoproterenol (ISO) increased the Ih current by a right-ward shift in the current activation curve. In current clamp, addition of ISO to spontaneously firing OAIs resulted in an increased firing frequency in OAIs. Analysis of the spike trajectory indicated that ISO preferentially affected the time course of the AHP and increased interspike interval decay rate. These effects of ISO on action potential generation are consistent with the increase in Ih current.

Distribution of the potassium channel subunit Kv2.1. One of the long-term goals of the group directed by McBain is to identify the molecular identity of the voltage-gated ion channels expressed on different subtypes of hippocampal inhibitory interneurons and to determine the role(s) played by individual potassium channel subunits. Kv2.1 belongs to the Shab subfamily of potassium channels and is widely expressed in principal neurons of the mammalian central nervous system, little is known, however, about its expression in the inhibitory interneurons of the CNS. Several neurochemical markers of interneurons, including the calcium binding proteins, parvalbumin, and calbindin, as well as the neuropeptide, somatostatin, have been found to be primarily associated with distinct subpopulations of GABAergic neurons in cortex and hippocampal areas allowing the subdivision of particular interneuron types. In the hippocampus, all parvalbumin, calbindin, and somatostatin containing interneurons possessed the Kv2.1 subunit. In the cortex, ~75% of parvalbumin-positive interneurons were also Kv2.1-positive. Calbindin and somatostatin positive cells throughout the entire brain appeared to be Kv2.1 positive. The use of confocal microscopy revealed the distribution of Kv2.1 to be punctate on the somata and dendritic trees of most interneurons in both the cortex and hippocampus. Thus Kv2.1 is perhaps the most widespread of all voltage-gated potassium channels studied to date. Its distribution on inhibitory interneurons together with its previous described widespread distribution on principal cells suggests that Kv2.1 may have a functional role in most central neuron types.

Antisense knockout of channels containing the Kv2.1 subunit. McBain's group hypothesized that channels formed by Kv2.1 subunits may give rise to a "generic" delayed rectifier current in most cells of the nervous system. Analysis of delayed rectifier currents from several classes of interneuron and principal cell types, however, revealed delayed rectifiers with little in common. The biophysical and pharmacological properties of this current type differed considerably from cell to cell suggesting that channels formed by Kv2.1 may coassemble with an as yet undescribed subunit or be subject to regulatory mechanisms peculiar to each cell type. Using antisense oligonucleotides to selectively knockout Kv2.1 subunit expression in cultured rat hippocampal slices, experiments were performed to elucidate the role played by Kv2.1 in central neurons. Western blot analysis revealed that Kv2.1 protein content decreased ~50% after antisense treatment. Consistent with this reduction, electrophysiological studies revealed that delayed rectifier currents in pyramidal neurons treated with antisense oligonucleotides were reduced in amplitude. Concomitant with the reduction in peak current amplitude was a 20mV positive-shift in the current activation point in antisense treated cultures. Antisense oligonucleotides prolong spike repolarization during electrographic seizure activity. In current clamp experiments, little effect was observed on the action potential waveform following antisense-oligonucleotide treatment under normal recording conditions. Elevation of the extracellular potassium ion concentration, a condition known to precipitate electrographic seizure activity in hippocampal slices, resulted in a dramatic broadening of the action potential in antisense treated pyramidal neurons. Action potential duration in antisense treated cells was 347% that of control cells. The half-width of the action potential however, was relatively unchanged, consistent with a specific role for the Kv2.1 current in the late phase of repolarization. These preliminary results suggest that under control conditions, the repertoire of currents which remain in antisense treated cells are sufficient to permit normal repolarization of the action potential waveform. In conditions where epileptogenic seizure events are observed, however, currents through Kv2.1 appear to be essential for complete spike repolarization and the prevention of overexcitation of the cell.

Arachidonic acid modulates the transient outward potassium current. Arachidonic acid (AA) has been shown to be an important second messenger and to modulate both neuronal excitability and synaptic transmission in a variety of preparations. Recent evidence has shown that AA selectively blocks transient "A-type" outward potassium currents generated by Kv4.2 homomeric channels expressed in oocytes. Currents through Kv4.2 channels have been proposed to underlie the principal transient current in the mammalian CNS. Consistent with this hypothesis is the observation that pyramidal neurons and a subpopulation of inhibitory interneurons express members of the Kv4 subfamily. To determine whether the transient current in both principal neurons and inhibitory interneurons was carried through Kv4.2-containing channels the modulation of transient A-type currents by AA and related compounds was investigated. AA dose-dependently blocked the transient current in CA1 pyramidal neurons with an IC50 of ~1mM. In addition, in the presence of AA a more rapid time constant of current inactivation was observed. AA selectively affected the voltage-dependence of inactivation by shifting the half inactivation potential ~-20mV. The effects of AA were mimicked by eicosatetraynoic acid (ETYA), a non-metabolizable AA analog; this suggests that the actions of AA are direct and do not require metabolism to another active product. Transient currents in outside-out patches from interneurons of the st. oriens-alveus also were sensitive to block by AA and ETYA, by a mechanism similar to that observed in principal cells. These data suggest that channels formed from the Kv4.2 subunit may underlie the transient current in both cells types.

Transient currents in basket cells are resistant to block by AA. A population of interneurons, "basket-cells", were characterized whose transient current, however, was 10-fold less sensitive to AA. The transient current despite being largely resistant to AA, was paradoxically modulated by the external potassium ion concentration ([K+]o). Reducing [K+]o , resulted in a marked attenuation of the transient current amplitude. In contrast, elevation of [K+]o resulted in a potentiation of the transient current. Such a mechanism of attenuation of K channel activity has been described previously for currents through channels formed by Kv1.4 subunits. Of particular interest, in experiments designed to monitor electrographic seizure activity arising from an elevation of [K+]o, action potential activity in these cells was largely resistant to epileptogenic activity and cells simply persisted in firing single action potentials, despite the large reduction in the potassium ion driving force. This observation can be explained by the resulting augmentation in the transient K currents in these cells during elevated potassium levels which then ensure that action potential repolarization will be complete, thus preventing the genesis of interictal activity. The above data underscore the potential importance of the roles played by Kv1.4 and 4.2 channels in prevention of epilleptogenic events in particular neuron types.

ION CHANNELS AND RECEPTORS IN GLIAL CELLS

Glial cells express a broad variety of ion channels and receptors. For a long time their functional role was poorly understood, but it now seems likely that the differential expression of receptors and channels is part of the normal developmental process in glia. Work in the Section on Molecular Neurobiology of Glia directed by ***Vittorio Gallo*** is focussed on the physiological role of glutamate receptors during glial development and on the regulation of glutamate receptor gene expression in the brain.

Growth factor-induced regulation of glutamate receptors in oligodendrocyte progenitor cells. Platelet-derived and basic fibroblast growth factors (PDGF and bFGF, respectively) regulate oligodendrocyte progenitor (O-2A) proliferation, migration and differentiation *in vitro* and *in vivo.* O-2A cells co-express both AMPA- and kainate-preferring glutamate receptor (GluR) subunits. Experiments in progress in collaboration with William Hayes (Department of Biology, Catholic University) have demonstrated that O-2A progenitor cells express AMPA receptor genes *in vivo*, as detected by double *in situ* hybridization. O-2A cells were studied in developing rat cerebellar cortex sections, and identified on the basis of PDGF-a receptor mRNA expression, a marker for oligodendrocyte progenitors *in vivo.* PDGF-a receptor-expressing cells were found to express also GluR1-4 mRNAs. The relative levels of expression of the 4 AMPA receptor subunits were similar. On the other hand, in O-2A cells cultured without growth factors, the subunit GluR1 was expressed at much lower levels than GluR2-4, suggesting that GluR1 expression *in vivo* may be under the control of g factors that are missing in the culture medium.

To address this Li Jin Chew analyzed the effects of PDGF and bFGF on the expression of GluR subunit mRNAs in cultured O-2A cells after 3 days of treatment with the growth factors. bFGF caused a 2-3 fold increase in steady-state mRNA levels for GluR1, 2, 3 and 4. PDGF did not modify by itself the mRNA levels for any of the AMPA subunits, but selectively potentiated the effects of bFGF on GluR1 mRNAs (5-fold increase). The kainate-preferring subunits GluR7, KA1 and KA2 mRNAs were increased (2-4 fold) by bFGF, but these effects were not modified by co-treatment with PDGF. Nuclear run-on assays demonstrated that PDGF+bFGF selectively increased the rate of GluR1 gene transcription (2.5 fold over control) within 20 hr after treatment of O-2A cells. RNAse protection assays showed that the two growth factors stimulated a selective 3-fold increase in GluR1 mRNA after 24 hr. Western-blot analysis showed that GluR1 protein levels were selectively increased (6-fold over control) by PDGF+bFGF. GluR2, 3 and GluR4 proteins were unaffected. In collaboration with Mayer's group, functional expression of AMPA receptors was assessed by electrophysiological studies. The whole-cell recording configuration was used, in combination with rapid application of the agonist AMPA to cultured cells. Cyclothiazide was used in combination to AMPA, in order to suppress receptor desensitization and accurately measure the amplitudes of agonist-generated currents. From the amplitude of agonist-generated currents, normalized for the cell capacitance, it was concluded that AMPA receptor current densities (pA/pF) were increased nearly 5-fold in cells treated with PDGF+bFGF, as compared to untreated cells. Further, AMPA receptor channels in cells treated with PDGF+bFGF were more sensitive to voltage-dependent block by intracellular polyamines, as expected from the robust and selective enhancement of GluR1 expression. This indicated that treatment with PDGF and bFGF increased the density of AMPA receptors lacking GluR2. In conclusion, our combined molecular and electrophysiological findings indicate that AMPA receptor function can be regulated by growth factor-induced changes in the rate of gene transcription.

Physiological role of glutamate receptors in oligodendrocyte development. In culture, O-2A cells differentiate to oligodendrocytes with a time schedule similar to that *in vivo*. We hypothesized that the neurotransmitter glutamate may regulate oligodendrocyte development through the activation of membrane receptors expressed early on during O-2A proliferation and migration. We used *in vitro* bioassays and soluble factors, which stimulate mitosis, motility, and maturation of O-2A cells, to reproduce the events that occur during CNS development. Previous studies demonstrated that AMPA receptor activation inhibits O-2A cell proliferation and prevents lineage progression to the pre-oligodendrocyte stage. We have now begun to study the mechanism of inhibition of O-2A cell proliferation by glutamate receptor agonists (kainate and AMPA). O-2A cell membrane depolarization by the alkaloid veratridine or by high (25-45 mM) extracellular K^+ for 24 hr inhibited O-2A cell proliferation stimulated by PDGF, bFGF or PDGF+bFGF. The two depolarizing agents also prevented O-2A cell lineage progression, as measured by counting the % of $O4^+$ cells (pre-oligodendrocytes) under different culture conditions. In O-2A cells cultured for 48 hr with growth factors, 45 mM K^+ (similarly to kainate and AMPA) significantly decreased the % of O-2A cells that became $O4^+$.

Cristina Ghiani has began to study whether the anti-proliferative effects of GluR agonists and depolarizing agents are due to a reversible arrest of O-2A cells in G1 phase of the cell cycle. In synchronized O-2A cells, kainate treatment at G1 strongly decreased the % of cells that entered S-phase, whereas treatment during S- or M-phase itself did not affect O-2A cell proliferation. In collaboration with Peter Knutson and Chris McBain, we determined that the voltage K^+ channel phenotype of O-2A cell changed during their development *in vitro*. In particular, delayed rectifier K^+ channels are highly expressed at the O-2A stage, but down-regulated in pre-oligodendrocytes. Conversely, inwardly rectifying K^+ currents were very small in O-2A cells, but up-regulated in pre-oligodendrocytes. This change in K^+ channel phenotype suggests that these channels may be involved in O-2A development. It was found that activation of GluRs in O-2A cells causes a reversible block of voltage-dependent delayed rectifier K^+ channels. These channels were also blocked by the selective K^+ channel blocker tetraethylammonium (TEA). Concentrations of TEA that caused a reversible block of these K^+ channels also strongly inhibited growth factor-stimulated O-2A cell proliferation. Additionally, the % of $O4^+$ cells was significantly decreased by TEA. These results indicate that inhibition of O-2A cell proliferation occurs through a glutamate receptor-mediated block of the delayed rectifier K^+ channels. This block is primarily due the increase of intracellular Na^+ ions caused by the opening of glutamate-gated channels, as shown by experiments in which extracellular Na^+ ions were substituted with N-methyl-D-glucose. It appears that Ca^{2+} ions are not directly involved in the block of K^+ channels and in the anti-proliferative effects described above, because: i) kainate- and TEA-induced K^+ channel block is Ca^{2+}-independent, and ii) the Ca^{2+} ionophore A23187 did not modify O-2A cell proliferation. Consistently with their anti-proliferative effects, both veratridine and 45 mM K^+ caused a reversible block of K^+ channels in O-2A cells.

Glutamate receptor-induced Ca2+ transients and CREB phosphorylation in oligodendrocyte progenitor cells. GluR activation causes phosphorylation of the transcription factor CREB in O-2A cells. Mario Pende demonstrated that stimulation of G-protein coupled (muscarinic) and tyrosine kinase (PDGF and bFGF) receptors also triggered CREB phosphorylation. The mechanism of CREB phosphorylation, in particular its Ca^{2+}-dependence and the intracellular signal transduction pathway, were further investigated. Anti-CREB antibodies selective for the 43 kDa Ser-133 phosphorylated form of CREB were used. CREB phosphorylation was stimulated by kainate, carbachol and bFGF. Increase of cAMP levels by forskolin caused only a weak induction of CREB phosphorylation. CREB phosphorylation by kainate and carbachol was rapid and transient (peak at 5 min), whereas the effects of PDGF or bFGF had a slower onset and remained constant for at least 30 min. In collaboration with Peter Simpson and James Russell, we studied the effects of kainate, carbachol and bFGF on intracellular Ca^{2+} transients. Kainate and carbachol stimulated transmembrane influx of the cation. Carbachol also triggered Ca^{2+} release from intracellular stores. bFGF responses were only displayed by 20% of the cells and their amplitude was several fold lower than the kainate or carbachol responses. The absence of extracellular Ca^{2+} abolished the kainate and bFGF responses, and eliminated the plateau component of the carbachol response. The intracellular Ca^{2+} chelator BAPTA abolished all responses. Omission of Ca^{2+} ions from the extracellular medium abolished kainate-induced CREB phosphorylation, strongly attenuated the effects of carbachol, and did not influence the effect of bFGF. Chelation of intracellular Ca^{2+} by BAPTA reduced the responses of all these stimuli.

Our experiments suggest that CREB phosphorylation stimulated by kainate and carbachol is dependent on transmembrane influx of Ca^{2+}, whereas the effects of bFGF depend on intracellular Ca^{2+}. Ca2+ ions directly influence the activity of many key regulatory enzymes, including protein kinases. Biochemical analysis of different protein kinase pathways (Ca^{2+}-calmodulin-dependent kinase, CaMK; protein kinase C, PKC; pp90 ribosomal S6 kinase, RSK; mitogen-activated kinase, MAPK), combined with the use of selective inhibitors demonstrated that CaMK and PKC are exclusively stimulated by Ca^{2+} influx, S6K exclusively by growth factors and RSK is stimulated by both types of signals. Consistently with previous findings that RSK is a downstream effector of the MAPK pathway, we determined that this pathway was responsible for phosphorylation of CREB triggered by kainate, carbachol and growth factors. In conclusion, the MAPK pathway can propagate membrane receptor signals to the nucleus in O-2A cells by inducing CREB phosphorylation.

Exon-intron organization of the rat glutamate receptor gene *GRIK5* and analysis of an intronic negative regulatory region. The gene *GRIK5* (glutamate receptor ionotropic kainate 5) encodes the kainate-preferring glutamate receptor subunit KA2. Fei Huang isolated and analyzed the structure of the gene *GRIK5*. Six overlapping DNA fragments containing the entire *GRIK5* gene were identified in a rat genomic library. *GRIK5* is a unique gene distributed over 54 kilobases of genomic sequence. *GRIK5* is composed of 19 translated exons and one untranslated exon, ranging in size from 54 to 944 bp. Exon 20 (944 bp) of *GRIK5* contains 423 bp of coding and 521 bp of 3' untranslated region (a TGA translational stop codon is present 424 nucleotides downstream of the intron-exon boundary). The total length of *GRIK5* coding region (3.78 kb) deduced form the genomic sequence is consistent with the 3.9 kb mRNA identified by Northern blot analysis of tissue and cultured neural cell mRNAs. Based on the previously cloned rat KA2 cDNA sequence, polyadenylation of *GRIK5* mRNA occurs 23 nucleotides downstream of a canonical signal sequence (AATAAA) in exon 20 (nucleotides 917-922 from the 5' end of exon 20). Analysis of *GRIK5* genomic sequence and comparison with the cloned rat and mouse KA2 cDNAs suggest that, differently from other kainate and AMPA receptor subunit genes, neither alternative splicing nor RNA editing of this gene occur.

Previous analysis demonstrated that 2 kb of *GRIK5* 5'-flanking sequence confers tissue-specific expression on a chloramphenicol acetyltransferase (CAT) reporter gene *in vitro* and *in vivo*. Fei Huang found that the first intron of *GRIK5* (3.4 kb) interrupts the 5' untranslated region. By using transient transfection assays in CG-4 and PC12 cells, it was demonstrated that the first intron of *GRIK5* inhibited transcription of the CAT gene driven by the 2 kb 5'-flanking region of *GRIK5*. Deletion construct analysis indicated that the negative regulatory element resides within 500 bp of the 3'-end of intron 1. The effect of the negative regulatory element on *GRIK5* transcription was orientation-, position- and distance-independent, therefore, displays the features of a silencer. This element also inhibited CAT transcription driven by the heterologous b-actin and SV40 promoters in HeLa and 3T3 cells. Finally, gel shift assays demonstrated that the 500 bp fragment of *GRIK5* intron 1 selectively bound nuclear proteins isolated from neural and non-neural cells. We conclude that regulation of transcription of the kainate receptor *GRIK5* gene results from an interplay between positive elements located in the promoter region and an element that displays features of a silencer residing within the first intron.

SECOND MESSENGERS AND SIGNALLING IN GLIA AND PINEAL CELLS

One focus of the Section on Neuronal Secretory Systems directed by ***James Russell*** concerns the cellular processes that control glial cell signalling in response to neuronal activity. This encompasses investigation of intracellular calcium metabolism and calcium signalling and cellular mechanisms of signal transduction for receptors involved in calcium based excitability.

The group also is involved in research to understand the regulation of cellular excitability of mammalian pineal cells.

It is now clear that glial cells monitor neuronal activity and respond to neuronal signals with a form of calcium based excitability. Although glial cells do not show electrically excitable properties similar to neurons, they participate in long distance signalling with propagated calcium waves within cells and between cells in networks. Neither the cellular mechanism of initiation and propagation of astrocytic calcium waves nor the precise physiological function of such signalling in the brain is currently known. During development, such signalling is thought to provide developmental cues for cell migration and path finding through trophic substances secreted by glial cells and regulated by cellular activity. This group has endeavored to understand the cellular mechanisms and processes that underlie agonist stimulated calcium wave initiation and propagation using the cultured astrocyte as a model system. Two major questions are under investigation. What are the cellular mechanisms that support long distance propagation of calcium signals in glial cells? Are there structural specializations to support these processes?

Intracellular calcium waves in glial cells. Astrocytes in tissue culture are an excellent model system in which to study intracellular calcium waves for a number of reasons. Chiefly, cultured astroglia grow as very thin sheets upon glass cover slips and spread out to extend processes. Furthermore, these cells are robust in culture and allow for experiments lasting many hours on the microscope stage when perfused with saline solutions. Using fluorescence microscopy of cells loaded with reporter dyes, it is possible to record the dynamics of calcium ion concentration changes with a high degree of spatial and temporal resolution.

Previous work from the group has described in detail the phenomenon of saltatory wave propagation in astrocytes following stimulation with neurotransmitters. Propagation of a calcium wave is achieved by enhanced calcium release at discrete release sites which give rise to highly localized calcium signals in cells. These units function as cellular wave amplification loci and were spread throughout the cell. These units can operate independently or be coupled by the diffusing calcium ions to generate the wave. The physical nature of this cellular specialization, ie. the calcium release site is unclear.

We hypothesized that a high density distribution of $InsP_3$ receptor channels ($InsP_3R$) on the endoplasmic reticulum (ER) could be present in the enhanced calcium release site. Carol Sheppard characterized the distribution of the different subtypes of $InsP_3Rs$ on the ER of astrocytes using immunocytochemical methods to determine if the wave origination and amplification loci possessed higher density of receptor channels. All three receptor subtypes were distributed throughout the endoplasmic reticulum in type 1 astroglia. There, however, were differences in the intensity of labelling by the antibodies in different regions of the cells. Regions of high calcium release during wave propagation was associated with high density of $InsP_3R2$. Cross correlation analysis provided a quantitative comparison of the pattern of $InsP_3R2$ distribution and the calcium release kinetics. In addition to the enhanced release sites, ample staining against $InsP_3R2$ was also observed in regions where the calcium release kinetics were several fold lower. This observation suggested that the distribution of the ER calcium release channels alone could not explain the specialized release sites in glial cells and prompted a search for cellular specializations other than $InsP_3R$ distribution.

Peter Simpson together with Carol Sheppard started work on the endoplasmic reticulum of both type 1 and type 2 astrocytes. They showed by immunocytochemistry that astrocytes express ryanodine receptors throughout the ER. Peter Simpson showed that these ryanodine receptor channels are functional and their activation can initiate calcium waves in different types of glial cells including oligodendrocytes. Furthermore, comparison of $InsP_3R$ mediated calcium waves and ryanodine receptor evoked waves in the same cells showed that in both cases the wave amplification sites were the same. This results is consistent with the idea that glial cells possess a single functional calcium pool which is specialized in certain regions of the cell as wave amplification sites. While it is possible that $InsP_3Rs$ and ryanodine receptors are colocalized at these specialized sites, an alternative or complementary explanation would be that the major factor or factors governing discrete calcium release sites is related to cellular characteristics other than the density of ER receptor channels alone.

We examined specializations in the ER and distribution of mitochondria in relation to calcium wave propagation and locally discrete wave amplification sites. Three observations are notable. First, similar to the distribution of $InsP_3R2$ and the ryanodine receptors, the SERCA type calcium pumps were also colocalized in the wave amplification sites. Second, the wave amplification sites were always associated with the presence of mitochondria such that no release sites were found at cellular regions where mitochondria were absent, and mitochondria were not found at sites where enhanced calcium release was not observed. Third, the endoplasmic reticulum in the release sites showed a highly specialized beaded accumulation of the intraluminal calcium binding protein calreticulin. These findings taken together support the view that the locally discrete calcium release sites which act as calcium wave amplification sites possess a number of specializations which include: High density distribution of $InsP_3R2$, ryanodine receptors and SERCA pumps on the ER. The presence of one or more mitochondria in close association with the ER membranes. A bead-like local condensation of calreticulin in the ER lumen. We propose that the cell assembles such a specialized calcium release machinery in order to support both locally discrete calcium signals as well as to support propagation of calcium waves over long distances.

Pineal cell excitability

Nissim Darvish set up a patch clamp rig in order to study ion channels that control membrane excitability in mammalian pineal cells. This approach was initially undertaken to characterize the particular cGMP gated nonselective cation conductances described in earlier work investigating the signal transduction of VIP receptors. Over the last year Darvish has succeeded in describing a number of unique ionic conductances in pineal cells. The major finding that resulted from this work is characterization of a novel mechanism of channel modulation via cyclic AMP and calcium ions. In the rat, circadian rhythm in melatonin is regulated by noradrenergic and neuropeptide inputs to the pineal via a cAMP and calcium dependent mechanisms. We discovered a large conductance (180 pS), voltage-dependent, non-selective cation channel on pineal cells with a novel mode of modulation by cAMP. PACAP, norepinephrine or 8BrcAMP cause a 10 fold increase in the open probability of the channel with a shift in voltage dependence towards zero from highly depolarized membrane potentials. The increase in open probability is followed by a change in current rectification properties, such that the channel is transformed from being inactive at rest to an inwardly rectifying cation conductance when activated by norepinephrine or PACAP, which depolarizes the cell. This channel is calcium insensitive, blocked by cesium and shows a permeability sequence: $K^+ > Na^+ > Li^+ > NH4^+$. This channel may support the long lasting stimulation necessary for circadian control of melatonin secretion mediated by neurotransmitters such as norepinephrine and PACAP.

ANNUAL BIBLIOGRAPHY

Chikwendu A, McBain CJ Two Temporally Overlapping "Delayed-Rectifiers Determine the Voltage-Dependent Potassium Current Phenotype in Cultured Hippocampal Neurons. J Neurophysiol (in press).

Du J, Zhang L,Weiser M, Rudy B, McBain CJ Developmental Expression and Functional Characterization of the Potassium Channel Subunit Kv3.1b in Parvalbumin-Containing Interneurons of the Rat Hippocampus. J Neurosci 1996;16: 506 - 18.

Fleck MW, Bähring R, Patneau DK, Mayer ML AMPA receptor heterogeneity in rat hippocampal neurons revealed by differential sensitivity to cyclothiazide. J Neurophysiol 1996;75:2322-2333.

Gallo V, Russell JT Excitatory aminoacid receptors in glia different subtypes for distinct functions? J Neurosci Res 1995; 42: 1-8.

Gallo V, Zhou JM, McBain CJ, Wright P, Knutson PL, Armstrong RC Oligodendrocyte progenitor cell proliferation and lineage progression are regulated by glutamate receptor-mediated K^+ channel block. J Neurosci 1996; 16: 2659-2670.

Holtzclaw L, Gallo V, Russell JT AMPA receptors shape Ca^{2+} responses in cortical oligodendrocyte progenitors and CG-4 cells. J Neurosci Res 1995; 42: 124-130.

Levi G, Gallo V Release of neuroactive amino acids from glia. In: Kettenmann H, Ransom BR, eds. Neuroglia. New York: Oxford University Press, 1995; 815-826.

Maccaferri G, McBain CJ Long-Term Potentiation in Distinct Subtypes of Hippocampal Non-Pyramidal Cells. J Neurosci 1996; 16: in press.

Maccaferri G , McBain CJ The Hyperpolarization-Activated Current (Ih) and its Contribution to Pacemaker Activity in Rat CA1 Hippocampal St. Oriens/Alveus Interneurons. J Physiol (Lond) 1996; in press.

McBain C J, Maccaferri G Synaptic Plasticity in Hippocampal Interneurons?: A Commentary. Canadian Journal of Pharmacology and Physiology 1996 (in press).

Partin KM, Mayer ML Negative allosteric modulation of wild type and mutant AMPA receptors by GYKI 53655. Mol Pharmacol 1996;49:142-148.

Partin KM, Fleck MW, Mayer ML AMPA Receptor Flip/Flop Mutants Affecting Deactivation, Desensitization and Modulation by Cyclothiazide, Aniracetam and Thiocyanate. J Neurosci (in press).

Steinhauser C, Gallo V News on glutamate receptors in glial cells. Trends Neurosci 1996; 19: 339-345.

Vyklicky L, Zemkova H, Vlachova V Copper modulation of NMDA responses in mouse and rat cultured hippocampal neurons. Eur J Neurosci (in press).

Yagodin S, Holtzclaw L, Russel JT Subcelluar calcium oscillators and calcium influx support agonist-induced caqlcium waves in cultured astrocytes. Mol Cell Biochem 1995; 149-150: 137-144.

Project Numbers - Annual Report 1996

LABORATORY OF CELLULAR AND MOLECULAR NEUROPHYSIOLOGY (LCMN)

ZO1 HD 00707-12	Pharmacological Studies of Synaptic Transmission In Vitro Mark L. Mayer, Ph.D.
Z01 HD 01205-04	Cellular and Synaptic Physiology of Hippocampal Interneurons Chris J. McBain, Ph.D.
Z01 HD 01206-03	Receptor Mediated Calcium Signalling in Glia and Neurons James T. Russell, D.V.M., Ph.D.
ZO1 HD 02000-05	Neurotransmitter Receptors in Glia Vittorio Gallo, Ph.D.

ZO1 HD 01207-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00707 12 LCMN

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pharmacological Studies of Synaptic Transmission In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	M.L. Mayer	Research Physiologist	LCMN, NICHD	12 months
Others:	K. Partin	IRTA fellow	LCMN, NICHD	10 months
	D. Bowie	Visiting Fellow	LCMN, NICHD	12 months
	M. Fleck	IRTA Fellow	LCMN, NICHD	12 months
	R. Bähring	Visiting Fellow	LCMN, NICHD	12 months
	C. Glasser	Chemist	LCMN, NICHD	10 months

COOPERATING UNITS *(if any)*

Dr. M. Benveniste, Sackler School of Medicine,
Tel Aviv University, Israel.

LAB/BRANCH

Laboratory of Cellular and Molecular Neurophysiology

SECTION

Section of Neurophysiology and Biophysics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.6	4.8	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This section investigates the functional properties of neurotransmitter receptors in the vertebrate CNS utilizing electrophysiological and molecular biological techniques. Receptor selective agonists and antagonists are applied by rapid perfusion to cells and membrane patches under voltage clamp. Preparations in use include recombinant glutamate receptors expressed in transfected mammalian cells and Xenopus oocytes, and native glutamate receptors generated in primary cultures of selected CNS cells. Kinetic analysis of the molecular basis of AMPA receptor allosteric regulation is being investigated by generation of mutants in an extracellular domain controlling sensitivity to potentiation by cyclothiazide and aniracetam. In glial progenitor cells regulation of expression of native AMPA receptors by the growth factors PDGF and bFGF was analysed by electrophysiological techniques. Increased rectification after treatment with PDGF and bFGF revealed selective expression of polyamine sensitive subunits. The mechanism of polyamine induced rectification was characterized using GluR6(Q) homomeric receptors. Spermine and philanthotoxin (PhTX) block from both the cytoplasmic and external faces of the channel; external spermine block is very weak, and for PhTX the rate of recovery from block increases with hyperpolarization. An Eyring rate theory model with an asymmetrical external barrier higher for PhTX than for spermine adequately reproduced these features.

Project Description:

Objectives: To characterize the functional properties of excitatory amino acid receptors. To investigate the mechanisms of neurotransmitter and drug action at glutamate receptors. To study the molecular properties of ion channels in neurons and glia, and to characterize mechanisms regulating neuronal and glial cell excitability. To characterize the role of glutamate receptors in synaptic transmission in the vertebrate central nervous system

Methods employed: Electrophysiological and molecular techniques are used to study neurotransmitter receptor function using a variety of preparations, primarily recombinant glutamate receptors transiently expressed in oocytes and transfected human embryonic kidney cells, as well as native receptors expressed in neurons and glial cells. Cotransfection with a reporter plasmid encoding a mutant jellyfish green fluorescent protein allows transfected cells to be identified by fluorescence microscopy prior to electrophysiological recording. Ion channels activated by excitatory amino acids are studied using various configurations of the patch clamp technique. Neurotransmitters and drugs are applied by rapid perfusion. A piezoelectric manipulator is used to achieve solution exchange in less than 1 ms for outside-out patches. For whole-cell recording an array of computer controlled valves and microperfusion tubes allows exchange on the 10 ms time scale. Simultaneous intracellular recording from pairs of synaptically connected neurons, and analysis of autaptic responses from neurons grown in microculture are used to study excitatory synaptic transmission. Computer analysis of the kinetic properties of agonist evoked membrane currents, single channel activity, synaptic release probabilities and the simulation of the gating and permeation properties of glutamate receptor channels plays an important role in the design and interpretation of our experiments.

Major findings: Three projects were undertaken this year: (1) Detailed kinetic analysis of a series of AMPA receptor GluRA mutations introduced at the Ser/Asn residue which is exchanged by alternative splicing and which in wild type receptors controls the action of cyclothiazide. (2) Characterization of the mechanisms underlying channel block of AMPA and kainate receptors by polyamines and by polyamine amide toxins. (3) Analysis of the effects of growth factors on AMPA receptor expression in glial progenitor cells.

Kinetic analysis of mutant AMPA receptors. Recent studies in our laboratory established that cyclothiazide, an allosteric modulator of glutamate receptor desensitization with complete selectivity for activity at AMPA versus kainate preferring receptors, preferentially modulates the flip versus flop splice variants of AMPA receptors. Sequence alignment of the flip/flop domains of each of the four AMPA receptor subunits (which are generated via alternative splicing of two modules each encoding 38 amino acids) reveals conserved substitution of just 7 amino acids between splice variants. These are arranged as shown below:

```
Consensus Flip    Thr-Pro-(8 AA)-Ser-(21 AA)-Lys-Asp-Ser-Gly-(2 AA)
Consensus Flop    Asn-Ala-(8 AA)-Asn-(21 AA)-Gly-Gly-Gly-Asp-(2 AA)
                                 ***
```

We showed by site-directed mutagenesis that the isolated serine residue shown by *** in the above alignment (S750 in GluRA) underlies the high sensitivity to cyclothiazide typical of wild type GluRAflip. In earlier work on native AMPA receptors we had found that in addition to blocking desensitization, cyclothiazide slowed AMPA receptor deactivation, and prolonged the decay of excitatory synaptic currents (EPSCs). The latter effect was similar to that produced by aniracetam, a weak regulator of AMPA receptor desensitization. The kinetic mechanism(s) underlying regulation of desensitization, deactivation, and EPSC decay by cyclothiazide and aniracetam are complex and poorly understood, but are important to address in view of suggestions that memory-and cognition enhancing drugs have AMPA receptors as their underlying target of action. By analysing with high temporal resolution responses recorded from both wild type and mutant AMPA receptors Kathy Partin and Mark

Fleck attempted to resolve the underlying mechanism of action of these drugs. These experiments revealed that substitutions at position 750 in GluRAflip to THR, LEU, TYR and VAL did not generate functional receptors; CYS and MET expressed poorly; ASP gave responses only in the presence of cyclothiazide; the remaining mutants generated robust whole-cell responses to glutamate both in the presence and absence of aniracetam and cyclothiazide, but with kinetics which differed from wild type. The only receptor for which desensitization was essentially blocked by cyclothiazide was wild type GluRAflip. For the remaining functional mutants the rate of onset of desensitization was slowed most for S750A (time constant 1690 ms); an intermediate response similar to that for wild type GluRAflop was observed for S750G (time constant 332 ms) and S750N (time constant 336 ms); for S750M, S750C and S750Q the rate of onset of desensitization in the presence of cyclothiazide was rapid (time constant 3 to 6 ms) and not significantly different from control. S750D was unusual in that it expressed no current under control conditions, but large currents in the presence of cyclothiazide; the decay in the presence of cyclothiazide was best fit by a double exponential (time constants 58 and 2857 ms).

To determine the role of position 750 in differential modulation by aniracetam, the desensitization kinetics of the same mutants assayed for modulation by cyclothiazide were analysed for sensitivity to 5 mM aniracetam, a concentration chosen on the basis of prior work on native AMPA receptors. For whole-cell responses aniracetam slowed the onset of desensitization for S750N (time constant 31 ms) to a greater extent than for GluRAflip (time constant 20 ms); the extent of modulation for S750N was similar to that for GluRAflop (time constant 32 ms) suggesting that an ASN residue at position 750 underlies the higher sensitivity of flop splice variants to aniracetam, while for cyclothiazide effective modulation requires a SER residue.

Mutations at SER750 control kinetics of deactivation and desensitization. Whole cell recording techniques lack sufficient resolution to determine the time course of channel closing which occurs following removal of agonist. This was measured with 1 ms applications of 10 mM glutamate to outside-out patches for GluRAflip, GluRAflop, S750N, S750Q and S750A. The subunits that were chosen for further study represented either naturally occurring isoforms; expressed a critical determinant of differential modulation by aniracetam and cyclothiazide (S750N); or exhibited altered desensitization kinetics for whole cell responses (S750A and S750Q). In patches we found that desensitization occurred even during a 1 ms application of glutamate, such that for different subunits the start of deactivation was fit well only for the later phase of the response after removal of agonist. The deactivation kinetics of S750A (time constant 0.97 ms) were significantly slower and those for S750Q (0.68 ms) faster than other subunits tested. There were no significant differences between GluRAflip and GluRAflop in deactivation kinetics. The deactivation kinetics of wild type GluRAflip are much faster than those of AMPA receptors in hippocampal neurons. Cyclothiazide slowed the rate of deactivation of GluRAflip by 2.4-fold, but did not modulate deactivation of GluRAflop. Surprisingly, in view of its weaker effects on desensitization, aniracetam was more effective than cyclothiazide at modulating deactivation, and produced a 4.1-fold slowing for GluRAflop, with a 2.2-fold slowing for GluRAflip.

Modulation of deactivation by cyclothiazide for S750N, S750A and S750Q followed the same trend as for modulation of desensitization in that clearly the SER residue permits the most efficacious slowing of deactivation (2.4 -fold); deactivation of S750A was slowed by 1.5-fold, whereas deactivation of S750N and S750Q was not affected. Aniracetam slowed deactivation for S750N (3.4-fold) and GluRAflop (4.1-fold) to a greater extent than the other subunits, similar to its effects on desensitization.

Thiocyanate modulation of AMPA receptor splice isoforms. Recent experiments on native and reconstituted AMPA receptors have shown that thiocyanate speeds up desensitization. To test for differential sensitivity of AMPA receptor splice isoforms to thiocyanate, we first compared whole cell responses to 500 _ M AMPA in the presence and absence of 20 mM thiocyanate. The effect of thiocyanate on GluRAflip was similar to that found in hippocampal neurons in that the peak was reduced about 20% and desensitization accelerated 1.4-fold. For GluRAflop, however, the peak was consistently increased by about 35%, and desensitization slowed. We also tested S750Q (resistant to cyclothiazide) and S750N (increased sensitivity to aniracetam) for modulation of desensitization by thiocyanate. For S750N the effect of SCN was similar to that for GluRAflop, whereas for S750Q desensitization was accelerated 1.4-fold. Although control responses for S750A desensitized almost twice as slowly as S750Q, thiocyanate accelerated the rate of onset of desensitization similarly for both mutants.

Kinetic modeling of modulation by aniracetam and cyclothiazide. A 12-state cyclic model for ligand-gated ion channels with binding of 2 molecules of agonist leading to conformational transitions to a single open state or to agonist bound desensitized states was used to simulate allosteric modulation of AMPA receptor gating. The model allows desensitization to occur only from closed states. Modulation by aniracetam similar to that observed for GluRAflop was achieved by slowing the closing rate constant 6-fold. This reproduced slowing of deactivation and desensitization . As would be expected from a cyclic model, slowing the closing rate constant changes accessibility to ligand bound desensitized states, which results in a slowing of the rate of onset of desensitization. This interpretation is consistent with our prior analysis of modulation of native receptors by aniracetam reporting an increased mean open time and the burst length with no change in single channel conductance. The model predicts a linear relationship between modulation of deactivation and modulation of desensitization by aniracetam, a correlation confirmed experimentally.

A kinetic scheme reproducing modulation by cyclothiazide for GluRAflip was achieved through "stabilization" of the agonist bound non-desensitized closed state immediately preceding the open state; this was achieved by slowing the rate of onset of desensitization 10,000-fold and by increasing agonist affinity for the RMA2 state 50-fold. Modulation of the onset of desensitization alone cannot account for the slowing of deactivation, which requires stabilization of the RMA2 state; to achieve slowing of deactivation it was not necessary to slow the closing rate constant. The model predicts very efficacious modulation of desensitization with much less modulation of deactivation, an effect observed experimentally for cyclothiazide modulation of all GluRA constructs studied. These models predict that for aniracetam, but not cyclothiazide, there should be a linear correlation between modulation of deactivation and desensitization. Simulated responses reproduce all these features. Because GluRAflip desensitizes very little in the presence of cyclothiazide it is difficult to assign a value for the "rate of onset of desensitization" as a measure of the efficacy of cyclothiazide; instead we measured the dissociation rate of cyclothiazide in the presence of glutamate (time constant16 s) and confirmed that a similar slow rate was achieved by the model (11 s). Simulated dose-response relationships with 1 mM glutamate as agonist yielded an EC50 for cyclothiazide of 1.0 _ M, and a shift in the EC50 of the glutamate peak response from 880 _ M to 27 _ M in the presence of 100 _ M cyclothiazide, substantially reproducing the prior results on native receptors.

Polyamine Block of Glutamate Receptor Ion Channels. Prior work by Derek Bowie revealed that the biphasic rectification of calcium permeable AMPA and kainate receptors results from ion channel block by cytoplasmic polyamines. At moderately positive membrane potentials polyamines enter and block the ion channel pore; with stronger depolarization the current increases again, most probably because polyamines themselves permeate through the channel. Robert B_ hring and Derek Bowie have now obtained direct evidence in support of this from analysis of the blocking action of two polyamine amide toxins which are derivatives of spermine: philanthotoxin 3,4,3 (PhTX) and 4-OH-phenyl-propanoyl-spermine (PPS). Both of drugs these contain spermine with one terminal amine linked via a peptide bond to a tyrosine residue; PhTX contains in addition an amide linked butyryl side chain which extends the width of the molecule along the face of the phenyl ring in the tyrosine residue. Depolarization from +50 to +100 mV produces a large increase in outward current for spermine but not for PhTX. CPK models of PPS and PhTX, when viewed along their most narrow axis, have widths of approximately 7_ (PPS) and 7.5 _ (PhTX). Analysis of conductance voltage plots for internal block by equieffective concentrations of these compounds reveals a 2-fold increase in conductance on depolarization from +50 to +100 mv for PPS, with no change for PhTX. This suggests that PPS but not PhTX is able to cross the membrane, indicating a diameter in the most narrow region of the pore between 7 and 7.5 _ .

Analysis of the blocking action of extracellular spermine and philanthotoxin revealed complex responses not predicted from the results obtained for internal block. For external spermine block occurred at a low affinity site and showed weak voltage dependence; equilibrium dose response analysis revealed that the Kd behaved as a U-shaped function of voltage, with a minimum around zero mV, such that spermine affinity decreased with either membrane potential depolarization or hyperpolarization over the range -80 to +80 mV. Conversely, for block by internal spermine the dissociation constant decreased e-fold per 20 mv depolarization over the range -80 to +40 mV. Analysis of conductance-voltage plots for block by 30 _ m external spermine, obtained using voltage ramp protocols, revealed pronounced hysteresis. When ramps were applied in a hyperpolarizing direction, from +150 to -100 mV, the conductance reached a minimum of approximately 55% of control at around 0 mV, and then increased with further hyperpolarization to around 75% of control at -100 mV. However, when ramps were applied in a depolarizing direction, from -100 to +150 mV the conductance decreased strongly with depolarization, reaching less than 10% of control at +40 mV, before increasing with further depolarization to the reach the same value recorded with hyperpolarizing ramps at +150 mV. The response for depolarizing ramps strongly resembled that for block by internal spermine, raising the possibility that influx of spermine had occurred during prior hyperpolarization. This was tested by voltage jump protocols with brief hyperpolarization from +40 mV, which revealed transient block of agonist activated current only when spermine was coapplied with agonist during membrane potential hyperpolarization. The extent and duration of transient block increased with hyperpolarization, prepulse duration, and external spermine concentration.

In contrast to the low affinity for block by external spermine, PhTX proved to be a potent blocker of GluR6 responses with an equilibrium dissociation constant at -80 mV of 57 nM. Voltage ramps, from +150 to -100 mV during application of PhTX revealed voltage-dependent block, with no relief on hyperpolarization as seen for spermine. However, analysis of equilibrium dose response curves for spermine block at different membrane potentials failed to reveal a strong increase in affinity with hyperpolarization over the range -20 to -80 mV, even though the affinity decreased 160-fold on depolarization from -20 to +40 mV. Although kinetic analysis of responses to spermine revealed an increase in rate of onset of block with hyperpolarization, the increase in block rate constant showed saturation at -ve membrane potentials, while the dissociation rate constant showed, for an externally applied external positively charged pore blocker, a paradoxical increase with hyperpolarization. Such results suggest that externally applied PhTX must also be able to pass through the pore on membrane potential hyperpolarization, but at rates much slower than those observed for spermine, presumably reflecting steric hinderance of the tyrosyl-butyryl substituted amine group in the most narrow part of the channel.

Glutamate Receptor Expression in Glial Progenitor Cells. The functional implications of GluR diversity in the brain are beginning to be elucidated, but the molecular mechanisms that control GluR expression are not yet understood. Experiments in Vittorio Gallo's laboratory established that the growth factors PDGF and bFGF selectively potentiate the expression of GluRA mRNA expression in O-2A glial progenitor cells. Nuclear run-on assays further demonstrated that PDGF + bFGF selectively increased the rate of GluR1 gene transcription, and western-blot analysis showed that GluR1 protein levels were increased 6-fold over control by PDGF+bFGF. Utilizing the whole-cell recording configuration, with agonist applied by fast perfusion, Mark Fleck compared the magnitude of AMPA-evoked currents in O-2A cells grown in low serum medium with those of O-2A progenitor cells grown in the same medium containing PDGF and bFGF. AMPA receptor responses were significantly larger in cells treated with PDGF+bFGF compared with untreated cells grown in low serum medium. Although in both culture conditions AMPA receptor current amplitude and cell capacitance varied between cells, the slope of the relationship between cell capacitance and AMPA -evoked current amplitude was much steeper for cells grown with PDGF+bFGF (0.54 pA/pF) than for untreated cells (0.08 pA/pF). Analysis of the range of steady-state current densities, derived by normalization to whole-cell capacitance, also revealed two dissimilar distributions with a mean current density approximately 4-fold higher in cells treated with growth factors compared with untreated cells.

Effects of growth factors on AMPA receptor subunit composition. To further determine whether the enhanced functional expression of AMPA receptors in PDGF+bFGF treated glial progenitor cells is selective for GluRA, as indicated by molecular analysis at the RNA and protein levels, the current-voltage relationship of AMPA receptor responses was examined in PDGF+bFGF-treated and untreated cells. A selective enhancement of the expression of GluR1 should either generate homomeric AMPA receptors assembled from GluRA, or dilute the relative contribution of GluR2 subunits to heteromeric AMPA receptors generated by coassembly with GluR1 and perhaps other subunits. In either case enhanced sensitivity to voltage-dependent channel block by cytoplasmic polyamines would be expected. Current-voltage relationships revealed pronounced rectification for PDGF+bFGF-treated but not untreated cells. The proportion of the AMPA activated conductance sensitive to voltage-dependent block by polyamines, estimated from the relationship 1 - (Gmin/G-80), where Gmin is the minimum conductance recorded over the membrane potential range +10 to +90 mV averaged 0.56 in PDGF+bFGF-treated cells, while in untreated cells the ratio was 0.27. By fitting Boltzman functions to conductance-voltage plots obtained from cells with well defined voltage-dependent responses to AMPA we were able to estimate dissociation constants for block by spermine in both untreated and PDGF+bFGF-treated cells. In the untreated cells selected for this analysis GV, the voltage-sensitive component of the response to AMPA, was 44% of GTotal; the remaining 14/23 cells showed too little voltage dependent block for accurate analysis. In the cells treated with PDGF+bFGF and selected for analysis GV was 78% of GTotal. The properties of GluRs are ultimately determined by the types of subunits expressed in the cell membrane to form functional ligand-gated channels. These experiments reveal that transcriptional regulation of AMPA receptor expression can selectively regulate the functional properties of native AMPA receptors.

Significance to Biomedical Research and the Program of the Institute. The role of glutamate receptors in mediating excitatory synaptic transmission in all parts of the brain is now widely appreciated, but far from completely understood. Experiments with molecular cloning have identified multiple families of receptor subunits, many of which exist in several splice variants. The functional role of this diversity is still largely unknown but provides rich potential for regulation during development, and for therapeutic manipulation. In addition to a basic signalling function regulating information flow across synapses, excitatory amino acids are known to markedly influence the development of neuronal circuitry; to participate in the genesis of abnormal electrical activity associated with disease states; and to contribute to the pathophysiology of stroke and cerebral anoxia. The profound changes in central nervous system development which occur following the experimental manipulation of glutamate receptor function in embryos and in juvenile animals points to an important role in regulating growth of the human nervous system during childhood. Recent experiments with selective NMDA and AMPA receptor antagonists suggest that drugs targeted at both subtypes of the glutamate receptor family have anticonvulsant activity, and show neuroprotection in animal models of stroke. In addition, AMPA receptor antagonists produced a profound amelioration of motor dysfunction in animals models of Parkinson's disease. Preliminary gene mapping experiments to locate glutamate receptors in human chromosomes suggest that abnormalities in their expression could underlie CNS diseases of previously unknown origin; a clearer understanding of this issue is likely to emerge as maps are constructed for more subtypes.

A basic understanding of the mechanism of action of excitatory synaptic transmitters, and factors controlling their release, will be needed to understand the cellular basis of the complex behaviors regulated by the family of glutamate receptor ion channels. Research in the laboratory is directed towards this goal.

Proposed course:

(1) Most native AMPA receptors contain the GluRB subunit. The functional properties of GluRB have been hard to study in isolation because this subunit has low conductance when expressed as homomers. To analyse the effect of the GluRB subunit on AMPA receptor gating we are constructing GluRB with a GLN residue introduced at the Q/R mRNA editing site in the pore forming region. This will allow GluRB to generate large responses like those for homomeric GluRA. We are also exchanging amino acids in the R/G mRNA editing site in the amino terminus of the flip/flop region. These mutants will be generated in both GluRB flip and flop backgrounds and expressed as homomers and also as heteromers assembled with GluRA. The kinetics of control responses to glutamate will be assayed in both outside-out patches and in whole-cell; in addition we will examine modulation of deactivation and desensitization by cyclothiazide and aniracetam.

(2) A series of GluR6 mutants has been constructed by neutralizing charged amino acids located in the cytoplasmic loop and pore forming elements located between the cytoplasmic ends of the 1st and 3rd membrane regions. In addition, amino acids of varying side chain size and polarity have been inserted in and around the Q/R site which regulates sensitivity to polyamines and Ca permeability in GluR6. These mutants will next be screened for functional expression. Sensitivity to channel block by cytoplasmic polyamines will be examined in outside-out patches in an attempt to understand the structural requirements for high affinity binding of polyamines.

(3) Polyamine-amide toxins, specifically derivatives of philanthotoxin (PhTX), are novel and potentially important molecular probes of ion channel structure. This is because their structure is highly polarized, with an amide linked tyrosine residue at one end of an otherwise intact spermine molecule. The tyrosine interferes with permeation through the channel due to its large size, while allowing the rest of the polyamine chain to enter the pore and sense the electric field. In principle it should be possible to use PhTX analogues with polyamine chains of different length, with amines spaced at various separations and perhaps even replaced by polar but neutral hydroxyl groups, as probes of the distance over which the electric field drops within the pore of glutamate receptors. Pilot experiments with selected compounds will be performed to test the feasibility of this approach.

Publications

Fleck MW, Bähring R, Patneau DK, Mayer ML. AMPA receptor heterogeneity in rat hippocampal neurons revealed by differential sensitivity to cyclothiazide. Journal of Neurophysiology 1996;75:2322-2333.

Partin KM, Mayer, ML. Negative allosteric modulation of wild type and mutant AMPA receptors by GYKI 53655. Molecular Pharmacology 1996;49:142-148.

Partin KM, Fleck MW, Mayer,ML. AMPA Receptor Flip/Flop Mutants Affecting Deactivation, Desensitization and Modulation by Cyclothiazide, Aniracetam and Thiocyanate. Journal of Neuroscience 1996;(In Press).

Vyklicky L, Zemkova H, Vlachova V. Copper modulation of NMDA responses in mouse and rat cultured hippocampal neurons. European Journal of Neuroscience 1996; In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01205-04 LCMN

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular and Synaptic Physiology of Hippocampal Interneurons.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	C.J. McBain	Senior Staff Fellow	LCMN, NICHD 12 months
Others:	G. Maccaferri	Visiting Fellow	LCMN, NICHD 12 months
	J. Du	IRTA Fellow	LCMN, NICHD 12 months
	K. Toth	Visiting Fellow	LCMN, NICHD 4months
	S. Keros	Pre-IRTA Fellow	LCMN, NICHD 12 months
	P. Knutson	Pre-IRTA Fellow	LCMN, NICHD 12 months

COOPERATING UNITS *(if any)*

Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary (Tamas Freund, Attila Gulyas).

LAB/BRANCH

Laboratory of Cellular and Molecular Neurophysiology

SECTION

Unit on Cellular and Synaptic Physiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.5	3.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The aim of this project is to characterize the properties of voltage gated channels and synaptic transmission of hippocampal inhibitory neurons in the developing brain and how these properties impact hippocampal function under both physiological and pathological conditions. Although much is known about their neurochemistry, their role in the local circuits and the basic electrophysiological properties of inhibitory interneurons, little is known about the specific ionic or ligand gated channels expressed on this highly divergent population of cells. A major part of our effort is to understand the ionic mechanisms which regulate the activity of these cells and how these mechanisms impact hippocampal function using patch clamp, immunohistochemical and molecular techniques. Our work over the past year has focused on particular populations of inhibitory neurons of the CA1 subfield. We have characterized the mechanisms of synaptic plasticity occurring on distinct population of inhibitory neuron and how changes in synaptic strength onto these neurons influences hippocampal function. In addition we have characterized the members of the Shaker family of potassium channels expressed on inhibitory neurons using both a combined electrophysiological and immunohistochemical approach. We have determined the roles of several voltage-gated currents in st. Oriens-alveus interneurons and their modulation by arachidonic acid and β-adrenergic receptor activation. Furthermore we have investigated how "knock-out" of these channels by antisense oligonucleotides impacts the physiological function of these cells.

Project Description

Objectives: The aim of this project is to characterize the properties of voltage gated channels and synaptic transmission of hippocampal inhibitory neurons in the developing brain and how these properties impact hippocampal function under both physiological and pathological conditions. Although much is known about their neurochemistry, their role in the local circuits and the basic electrophysiological properties of inhibitory interneurons, little is known about the specific ionic or ligand gated channels expressed on this highly divergent population of cells. A major part of our effort is to understand the ionic mechanisms which regulate the activity of these cells and how these mechanisms impact hippocampal function. Furthermore, experiments to determine whether interneurons of the rodent hippocampus possess an NMDA-receptor mediated form of long-term potentiation were also undertaken.

Methods Employed: Whole cell patch clamp studies under current and voltage clamp configurations have been performed from visually identified inhibitory neurons of the st. oriens /alveus, st. lacunosum-moleculare and stratum pyramidale, maintained in hippocampal slices from neonatal animals. In addition outside-out macroscopic patches have been excised from the soma of interneurons held in the slice preparation in order to provide an accurate estimation of the activation and inactivation profiles of novel potassium channels expressed on these cells. Simultaneous whole cell patch clamp from interneurons and pyramidal neurons together with field recordings have been made in studies observing the role of interneurons in synaptic plasticity. Perforated patch techniques are now employed in studies investigating long-term potentiation in three different subtypes of interneuron. Combined electrophysiology, morphology and immunohistochemistry techniques are used on single inhibitory cells in the slice preparation to correlate interneuron physiology with the expression of particular potassium channel subunits. Antisense oligonucleotide technology is being used to "knock-out" the expression of specific members of the Shaker family of potassium channels.

Major Findings:

I. A Lack of Long Term Potentiation in Hippocampal Inhibitory Interneurons.

The hypothesis that excitatory synapses onto hippocampal inhibitory interneurons undergo forms of synaptic plasticity similar to that observed in principal pyramidal neurons has received much attention in the last few years. No general consensus exists however, concerning the presence (or absence) of long term potentiation and depression in non-pyramidal neurons. A large source of this disagreement stems from the experimental paradigms chosen to elicit synaptic plasticity in the various studies, since the vast majority of induction protocols previously used have not permitted the separation of plasticity occurring on principal cells, which then propagates to the inhibitory neuron, from possible direct plasticity on the interneurons themselves. To determine whether any interneuron subpopulation within the CA1 subfield did indeed posses NMDA receptor-dependent forms of cellular plasticity **Gianmaria Maccaferri** used a protocol which combines depolarization of the neuron under study with low frequency stimulation (1Hz, 1 minute) i.e. "pairing" to elicit direct forms of plasticity. This protocol is followed some 20 - 30 minutes later with a tetanic stimulation paradigm which will elicit synaptic plasticity in a large population of pyramidal neurons which can then passively propagate onto the interneuron of study. By using this combination of stimulation paradigms we characterized both cellular and passively propagated forms of plasticity on a variety of interneuron subpopulations.

Interneurons of st.oriens/alveus.

Two types of st. oriens/alveus interneurons received passive propagation of synaptic potentiation via the recurrent collaterals of CA1 pyramidal cells, but neither subtype possessed direct cellular plasticity. One interneuron class of the st. oriens-alveus, the so-called "OA-LM" cell, was primarily activated in a feedback manner, i.e. by the recurrent collaterals of the CA1 pyramidal neurons with little evidence for direct activation by afferents from CA3 pyramidal neurons. An example of the morphology of this class of interneuron is shown in Fig1 (panel A). The soma and the dendritic tree are restricted to st. OA, while the axonal plexus selectively innervates st. lacunosum-moleculare. The excitatory synaptic potentials recorded from these cells show a temporal delay when compared to the peak of the simultaneously recorded extracellular field population spike (fPS) in st. pyramidale (Fig1 B), consistent with their predominant excitation arising from CA1 pyramidal cells and not the Schaffer collaterals i.e. pure feedback activation. Consequently, even in the absence of a direct interneuron plasticity, these cells are influenced *indirectly* by protocols eliciting synaptic plasticity in CA1 pyramidal cells. In fact, the pairing protocol resulted in a long lasting depression at synapses onto the st.oriens interneuron (C2). Since the pyramidal neurons illustrated in C1 are not depolarized during the 1Hz stimulus paradigm, synaptic potentials onto these cells are depressed. Thus the activity observed in the pyramidal neuron population is simply mirrored in the activity of the single interneuron. Likewise, following the tetanic protocol (indicated by the arrow in C1 and C2) both the pyramidal neuron field recording and the single interneuron both show LTP. These data demonstrate that the synaptic plasticity recorded in the st. OA interneuron occurs, not at the excitatory synapses onto the inhibitory neuron *per se* but at the excitatory synapses of pyramidal neurons. The change in strength of the pyramidal neuron afferent output then propagates to the interneuron resulting in a network form of synaptic plasticity i.e. passive propagation, without a change in excitatory synaptic *strength* onto the interneuron.

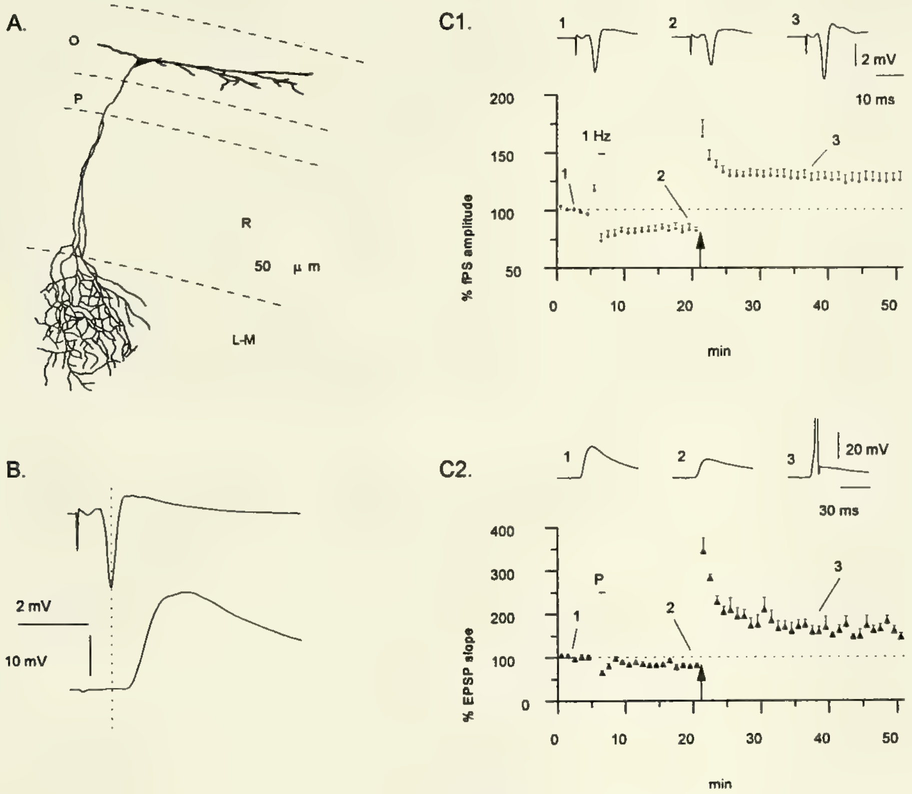


Figure 1.

Interneurons of St. Radiatum.

In st. radiatum, two distinct classes of cells were observed: st. radiatum interneurons which showed neither direct nor propagated forms of synaptic plasticity, and "giant-cells" whose EPSPs were robustly potentiated following a pairing protocol. This potentiation is similar to the LTP described in pyramidal cells and its induction requires NMDA receptor activation. Thus, a large heterogeneity of synaptic plasticity exists in morphologically distinct neurons and suggests that complex changes in the CA1 network properties will occur following the induction of LTP.

II. Identification of the stratum radiatum giant cell: interneuron or displaced pyramidal neuron?

Prompted by the unique properties of the st. radiatum "giant-cell" type, **Katalin Toth** investigated further the morphological and physiological properties of these cells to determine whether these cells were indeed an as yet undescribed interneuron of st. radiatum or a CA1 pyramidal neuron whose cell body is located out of the principal cell body layer. Typically, these cells possessed a large, triangular shaped cell body (~30µm) with its apex directed towards st. pyramidale. The dendritic tree was extremely elaborate, branching immediately away from the cell body in two main dendritic shafts, which could extend to, and cross, the hippocampal fissure. In contrast only a relatively restricted dendritic tree emerged from the apical end of the soma.

Whole cell recordings made from these neurones revealed cells with a stable resting membrane potential, (~-53mV) which displayed little or no spontaneous action potential activity. Injection of a positive current pulse elicited trains of action potentials which showed marked firing accommodation. With negative current pulses, a voltage sag was observed, consistent with the presence of the anomalous rectifier current (Ih). Action potential durations were quite broad; half width = 2.6 ± 0.6ms, and was usually followed by an afterdepolarization-afterhyperpolarization sequence. All these properties more closely resemble principal cells than the properties usually demonstrated by inhibitory interneurons.

To answer the question of whether "giant-cells" are truly a new class of inhibitory interneuron or alternatively is an excitatory pyramidal cell located in st. radiatum, electron microscopic analysis of the synaptic contacts made by this neuron was required. Inhibitory cells usually form symmetric synapses onto their target cells and possess dendrites which are spine-free, in contrast, excitatory principal cells make asymmetric synapses with their targets and have dendrites covered with spines. To this end we established a collaboration with Dr Tamas Freund's laboratory in Budapest , Hungary. Dr. Attila Gulyas provided unequivocal electron microscopic evidence that the giant-cell of st. radiatum was in fact a novel form of pyramidal neuron located in the st. radiatum.

In cells where the pairing protocol had successfully induced long-term potentiation (150.2 ± 17.3% over control ($p<0.05$), also see above), subsequent electron microscopy revealed that cells made asymmetric synapses onto target cells and spines were found on the dendritic shafts of the cell, indicating that these cells are excitatory even though the location of their cell body is atypical. That the synapses made by the giant-cells form asymmetric contacts with their target reveals that the synapses are excitatory in nature. Likewise, synapses made onto the giant cell occur on specialized dendritic spines which had evaded our detection at the light microscopic level. These data strongly suggest that the giant cell is indeed a principal neuron type whose cell body is placed out of st. pyramidale.

Since the giant cell of st. radiatum was the only cell type in which robust LTP was observed, which is in fact an excitatory pyramidal neuron, allows us to conclude that inhibitory interneurons of the mammalian hippocampus do not possess an NMDA-dependent form of long term potentiation.

III. The hyperpolarization activated current and its role as a pacemaker in st. oriens-alveus interneurons.

Despite the fact that interneurons constitute only about the 15% of the total hippocampal cell population, their role in the regulation of excitability is believed to be crucial. The intrinsic electrophysiological properties of these cells play an important role in the generation of their spontaneous activity. Under current clamp conditions in normal ACSF, regular spiking activity is commonly observed in OAIs. The frequency of spontaneous firing is unaffected by antagonists of AMPA and NMDA receptors, suggesting that rhythmic activity is independent of normal excitatory synaptic drive and is driven by intrinsic conductances.

Among the many different kind of voltage-operated conductances which are present in these interneurons, the hyperpolarization-activated current (Ih) is particularly interesting in this respect. In fact, Ih has been shown to be involved in the regulation of the firing pattern of thalamic neurones, is present in many different neuronal preparations, and was firstly described as the current responsible for spontaneous cardiac pacemaking. In order to study the basic properties of this current in st. oriens-alveus inhibitory neurons, **Gianmaria Maccaferri** has made whole-cell and perforated-patch voltage-clamp recordings from visually identified interneurons of st. oriens-alveus in the hippocampal "in vitro" slice preparation.

Kinetic and Pharmacological Properties of Ih

Ih was activated as an inward current by hyperpolarizing voltage pulses in the range -60 to -130mV. The activation kinetic was very slow (τ = ~200ms at -130mV) and no inactivation properties were detected. The threshold for activation was ~ -60mV, and was saturated at ~ -130mV and had a mid-activation point at ~-90mV. Consistent with its mixed ionic nature (Na^+ and K^+), its reversal potential was ~-30mV. The activation kinetics were fit by exponential functions and were found to be voltage dependent. At -70mV currents were fit by a single exponential of τ=2.8sec. while at -120mV two exponentials were required of τ_1=186ms (47.7±2.6%) and τ_2=1.04s (52.3±2.6%). Complete blockade of the Ih conductance could be achieved after cell exposure to external Cs^{2+} (0.25-5mM) or the specific Ih antagonist Zeneca ZD7288 (100μM), but was insensitive to Ba^{2+}.

Ih Modulates Spontaneous Firing in OAIs

To test whether I_h contributed to the AHP trajectory of spontaneously firing OAIs and/or played a role in the intrinsic pacemaking mechanism, we applied ZD7288 to interneurons displaying spontaneous firing activity Application of ZD7288 attenuated the spontaneous firing frequency. While ZD7288 had a minimal effect on the action potential waveform properties, suggesting a high degree of selectivity for the I_h current, when individual spikes were examined on a slower time scale it was apparent that the attenuation of the firing frequency resulted from a prolongation of the interspike interval. ZD7288 routinely decreased the slope of the depolarizing ramp leading to action potential threshold, analogous to the block of the voltage sag seen in response to electrotonic current injection. The decrease in the spontaneous firing frequency was not correlated, however, either to the initial firing rate or level of polarization. The action of ZD7288 reported here are similar to data from spontaneously beating sino-atrial node myocytes.

β-Adrenergic Agonists Increase Spontaneous Firing by Blocking Ih

Under voltage clamp conditions, Ih was modulated by β-adrenergic receptor agonists. Both norepinephrine (NE) and isoproterenol (ISO) increased the Ih current by shifting the activation curve to the right. In current clamp addition of ISO to spontaneously firing OAIs resulted in an increased firing frequency in OAIs. Analysis of the spike trajectory indicated that ISO preferentially affected the time course of the AHP and the rate of decay of the interspike interval These effects of ISO are consistent with the increase in the Ih current by ISO. Concomitant with the increased firing frequency was a decrease in the membrane input resistance which could be attributed to an increased inward current, presumably Ih. Consistent with a larger Ih current was the observation of the increased rate of depolarization on the "anode-break" and the shorter time to the first action potential compared to control.

These experiments reveal the presence of the hyperpolarization-activated current in st. oriens-alveus interneurons and demonstrate its role in the spontaneous firing pattern.

IV. Distribution of the potassium channel subunit Kv2.1 in parvalbumin, somatostatin and calbindin containing interneurons of the mammalian CNS.

Kv2.1 belongs to *Shab* subfamily of the Shaker class of voltage-gated potassium channels and encodes for a delayed-rectifier type of current in transfected cells. Kv2.1 is widely expressed throughout the mammalian central nervous system. While the distribution of Kv2.1 in most principle neurons of the CNS is well described , little is known about its expression in the inhibitory interneurons of the CNS. Several neurochemical markers of interneurons, including the calcium binding proteins, parvalbumin, and calbindin, as well as the neuropeptide, somatostatin, have been found to be primarily associated with distinct subpopulations of GABAergic neurons in cortex and hippocampal areas. Their expression in distinct subsets of interneuron classes, therefore, provides an ideal tool to further characterize the expression and physiological functions of Kv2.1 expressed in these cells.

Double immunostaining of Kv2.1 with either parvalbumin, calbindin, or somatostatin were examined in tissue sections derived from adult rat brain by **Jing Du**. In the hippocampus, all parvalbumin, calbindin, and somatostatin subpopulations of interneurons were found to express the Kv2.1 subunit. In the cortex, ~75% of parvalbumin-positive interneurons were also Kv2.1-positive, among which 30% were only weakly Kv2.1 positive. Both calbindin and somatostatin-immunoreactive interneurons in the cortex also contain the Kv2.1 subunit. However, ~50% of somatostatin-immunoreactive interneurons were only weakly Kv2.1 positive. Calbindin and somatostatin positive cells thoroughout the entire brain appeared to be Kv2.1 positive. Likewise while the majority of parvalbumin positive cells contain Kv2.1, a small number of parvalbumin-positive cells in the olfactory bulb and reticular system were Kv2.1 negative.

The use of confocal microscopy revealed the distribution of Kv2.1 to be punctate on the somata and dendritic trees of most interneurons in both the cortex and hippocampus. Electron microscopic studies are in progress to determine the subcellular distribution of the Kv2.1 subunit in interneurons. Thus Kv2.1 is perhaps the most widespread of all voltage-gated potassium channels studied to date. Its distribution on inhibitory interneurons together with its previous described widespread distribution on principal cells suggests that Kv2.1 may function as the generic delayed rectifier in most cell types.

V. Antisense knockout of channels containing the Kv2.1 subunit.

Since the potassium channel subunit Kv2.1 is widely expressed throughout rat central nervous system we hypothesized that channels formed by this subunit may give rise to a "generic" delayed rectifier current in most cells of the nervous system. Analysis of delayed rectifier currents from several classes of interneuron and principal cell types from data obtained from this lab and others, however, revealed delayed rectifiers with little in common. The biophysical and pharmacological properties of this current type differed considerably from cell to cell suggesting that channels formed by Kv2.1 may coassemble with an as yet undescribed subunit or be subject to regulatory mechanisms peculiar to each cell type. Previous work has demonstrated that Kv2.1 expression can be upregulated by NGF in PC12 cells and that channel function is regulated by a PKA phosphorylation mechanism.

Antisense oligonucleotides attenuate the delayed rectifier current in cultured pyramidal neurons

Kv2.1 channel function has been primarily studied in transfected or mRNA microinjected systems, while no studies of native channels containing Kv2.1 having been performed. **Jing Du** initiated an investigation into the turnover and function of native Kv2.1 channels in hippocampal neuron using antisense oligonucleotide technology. To determine the functional role of Kv2.1-containing channels in pyramidal neurons, antisense oligonucleotides were designed to selectively knockout Kv2.1 subunit expression in cultured rat hippocampal slices. Cultured hippocampal slices were incubated with appropriate antisense (or control scrambled) oligonucleotides directed against Kv2.1 for 5 days. Western block analysis revealed that total Kv2.1 protein content decreased ~40% after treatment with antisense compared with scrambled or untreated controls. Consistent with this reduction, preliminary electrophysiological studies revealed that delayed rectifier currents in both whole-cell and outside out patches from pyramidal neurons treated with antisense oligonucleotides were reduced in amplitude by 36 and 52% respectively when compared to control treated pyramidal neurons. Concomitant with the reduction in peak current amplitude was a 25mV positive-shift in the current activation point in antisense treated cultures. Transient current amplitudes remained unchanged in cultures treated with Kv2.1 antisense treated cultures, consistent with a selective reduction in the delayed rectifier currents by the antisense treatment.

Antisense oligonucleotides prolong spike repolarization during electrographic seizure activity.

In current clamp experiments, under control conditions little effect was observed on the action potential waveform following antisense-oligonucleotide treatment. Elevation of the extracellular potassium ion concentration, a condition known to precipitate electrographic seizure activity in hippocampal slices, resulted in a dramatic broadening of the action potential in antisense treated pyramidal neurons. Action potentials duration in antisense treated cells were 347%,when compared to action potential in control untreated slices. The half-width of the action potential was relatively unchanged in antisense treated cells consistent with a role for the delayed rectifier in the late phase of repolarization. These preliminary results suggest that under control conditions the repertoire of currents which remain in antisense treated cells are sufficient to permit normal repolarization of the action potential waveform. In conditions where electrographic seizure events are observed, however, currents through Kv2.1 appear to be essential for complete spike repolarization.

VI. Arachidonic acid modulates the transient outward potassium current in pyramidal neurons and st. oriens-alveus interneurons but not basket cells.

One of the long-term goals of this laboratory is to identify the molecular identity of the voltage-gated ion channels expressed on different subtypes of hippocampal inhibitory interneuron and the principal pyramidal neurons and the roles played by individual members of the Shaker family of potassium channels. Arachidonic acid (AA) strongly modulates both neuronal excitability and synaptic transmission in a variety of preparations. Recent evidence has shown that AA selectively blocks transient "A-type" outward potassium currents through Kv4.2 homomeric channels in oocytes. Currents through other members of the Shaker family of potassium channels were largely resistant to block by AA. Currents through Kv4.2 channels have been proposed to underlie the principal transient current in the mammalian CNS based on in situ hybridization patterns and hybrid arrest of Kv4.2 translation from whole brain mRNA. Consistent with this hypothesis is the observation that pyramidal neurons and a subpopulations of inhibitory interneurons express members of the Kv4 subfamily. To determine whether transient currents in principal neurons and various subtypes of inhibitory interneuron was carried through channels formed by Kv4.2 **Sotiros Keros** investigated the modulation of transient A-types currents by AA and related compounds. Using outside out patches from pyramidal neurons in both organotypic hippocampal slice cultures and conventional hippocampal in vitro slices Sotirios demonstrated that AA dose-dependently blocked the transient current in CA1 pyramidal neurons with an IC_{50} of ~1μM. At a test potential of +40 mV AA (10μM) blocked 70% of the transient current charge transfer. In addition a more rapid time constant of current inactivation was observed in the presence of AA. AA selectively affected the voltage-dependence of inactivation by shifting the half inactivation potential ~20mV in the hyperpolarizing direction. AA was without effect on the activation profile of the transient current.. The effects of AA were mimicked by eicosatetraynoic acid (ETYA), a non-metabolizable AA analog. ETYA (10μM) blocked 75% of the transient current charge transfer and like AA accelerated the rate of transient current inactivation. Transient currents in outside-out patches from interneurons of the st. oriens-alveus also were sensitive to block by AA and ETYA. The mechanism of block by AA and ETYA in these cells was identical to that seen in pyramidal neuron transient current suggesting that Kv4.2 or a related subfamily members underlie the transient current in both cells types.

Transient currents in basket cells are resistant to block by AA

Interestingly, we identified a population of interneurons whose cell bodies lie close to st. pyramidale i.e. "basket-cells", whose transient current was 10-fold less sensitive to AA. In these interneurons the transient current despite being largely resistant to AA, was paradoxically modulated by the external potassium ion concentration ($[K^+]_o$). Removal of $[K^+]_o$, resulted in a marked attenuation of the transient current amplitude by ~40%. In contrast elevation of $[K^+]_o$ resulted in a potentiation of the transient current. These observations are in contrast to what would be predicted from a simple change in the driving force for K^+. Such a mechanism of attenuation of K channel activity, however, has been described previously for currents through homomeric channels formed by Kv1.4 subunits. Consistent with the currents observed in the present experiments arising from channels formed by Kv1.4 is the recent report that Kv1.4 expression is restricted to a small population of cells whose cell bodies lie adjacent to the CA1 pyramidal cell layer, consistent with their being inhibitory interneurons. Of particular interest, in experiments designed to monitor electrographic seizure activity arising from an elevation of $[K^+]_o$ action potential activity in these cells was largely resistant to electrographic activity and simply persisted in firing simple single action potentials, despite the large reduction in the K driving force. This observation can be explained by the resulting augmentation in the transient K currents in these cells during elevated potassium levels which then ensure that action potential repolarization will be complete, thus preventing the genesis of interictal activity.

These data underscore the subunit heterogeneity of channels underlying the transient A-type current in pyramidal neurons and two distinct types of interneuron.

VII. Agents which prevent oligodendrocyte proliferation modify voltage gated potassium channel function.
In collaboration with Vittorio Gallo's laboratory, we had previously shown that oligodendrocyte (O-2A) proliferation and differentiation in culture, was inhibited, in a dose dependent manner, by the glutamate receptor agonist, kainate. GluR agonists were found to have no effect, however, on cells that had been allowed to differentiate into pro-oligodendroblasts (O4+). More recently, evidence obtained by Gallo's lab has shown that treatment of cultures with either high extracellular potassium, retinoic acid, or the K^+ channel blocker tetraethylammonium (TEA) exhibit a similar inhibition of O-2A proliferation and differentiation. In a continuing collaboration with Gallos lab (see Project Report # Z01-HD-02000-04) **Peter Knutson** has furthered these observations by making a detailed investigation of the physiological properties and the regulation of voltage-gated potassium channels on these cells following treatment with a variety of anti-proliferative agents.

Proliferating O-2A cells possess a repertoire of voltage-dependent potassium currents distinct from differentiated O4+ cells.

Using whole-cell patch clamp recording techniques, O-2A cells were observed to possess a relatively depolarized resting membrane potential (RMP) near -35mV, when compared to O4+ cells (RMP = ~-80mV). O-2A cells expressed several different voltage-dependent potassium channels. Currents in all cells were dominated by two delayed-rectifier-type K^+ currents, while 64% possessed an additional, rapidly inactivating, transient A-type current. Delayed rectifier currents were activated at membrane potentials near -40mV, and showed half-activation at -23mV and +16mV In addition most cells possessed a small amplitude, inward rectifying K^+ current. In contrast differentiated $O4^+$ cells expressed only a large inward rectifying K^+ current, on the order of 3 times that seen in O-2A cells. In addition no evidence for either type of outward potassium current was observed in $O4^+$ cells

Experiments were then performed to investigate the regulation of the delayed rectifier potassium currents present in the O-2A cells by the various anti-proliferative agents. Initial experiments showed that currents were strongly blocked by acute application of TEA and kainate, in the concentration range shown to block cell proliferation. Acute application of kainate and TEA on O-2A cells reduced the delayed rectifier K^+ current by 49%, and 75% respectively at a test potential of +70mV. No block was observed when kainate was applied in the presence of the glutamate receptor antagonist DNQX, or when NMDG was substituted for Na^+ in the extracellular solution. This suggests that the Na^+ influx following GluR activation was responsible for the block of the delayed rectifier current. In addition acute application of high extracellular K^+ (45mM) attenuated outward K^+ currents by 40% as was predicted from a change in the K^+ driving force. In addition, acute application of retinoic Acid, a vitamin A derivative, selective reduced the delayed rectifier current by 56%.

In order to elucidate how these agents might regulate proliferation and differentiation we tested cells which had been chronically cultured (24 hours) in the presence of the previously discussed agents. Chronic kainate application had no long term effect on the native outward K^+ currents. Chronic retinoic acid application caused a reduction (29%) in the delayed rectifier current, similar in magnitude to that seen with acute treatment. Chronic culture in 45mM $[K^+]_o$ potentiated all outward K^+ currents by 33%, in addition to upregulating the inward rectifying K current, demonstrating a distinct long term plastic change in K^+ channel function.

The anti-proliferative effects of both elevated external K$^+$, retinoic acid and GluR activation point towards a mechanism involving the regulation of K-channel function in O-2A cells. However, these data suggest that in O-2A cells, no single anti-proliferative agent resulted in currents which mimicked the phenotype observed in differentiated O4+ cells.

Proposed Course

1. Regulation of excitatory synaptic transmission onto st. oriens/alveus interneurons by mGlur7
Recent evidence suggests that synaptic transmission onto st. oriens/alveus "horizontal" interneurons may be regulated by an action of synaptically released glutamate onto metabotropic receptor subtypes, mGluR7. The demonstration that mGluR7 is restricted to the presynaptic grid of excitatory terminals only on somatostatin/mGluR1 containing interneurons suggests a unique form of autoregulation of this inhibitory cell type. We will perform experiments to test the hypothesis, that excitatory synaptic transmission onto st. oriens/alveus interneurons is regulated by mGluR7 receptor activation. In addition we will investigate whether long-term forms of plasticity can be observed in these cells following the blockade of mGluR7 receptors. Such experiments may shed light on the lack of any NMDA-dependent form of postsynaptic potentiation on these cells as desribed in the above project report.

2. Presynaptic forms of LTP expressed on interneurons of the st. lucidum
Since all interneurons subtypes tested within the CA1 subfield lack an NMDA-dependent form of long-term potentiation or depression, experiments will be performed on interneurons of the CA3 st lucidum to reveal whether pre-synaptic forms of LTP exist in these cells. Our data suggest that interneurons of the CA1 subfield, despite possessing NMDA receptors, may lack some essential second messenger component required for the formation of postsynaptic LTP. In contrast, plasticity at the mossy fiber terminals of the CA3 subfield however has been shown to be entirely pre-synaptic in the Guinea-pig and do not require activation of the postsynaptic elements. To determine whether the same mossy fibers which contact CA3 pyramidal neurons which also make contact with st lucidum cells possess presynaptic LTP, we will perform experiments on hippocampal slices prepared from Guinea-pig hippocampus. Anatomical evidence suggests that mossy fibre terminals onto st. lucidum interneurons markedly differ from the equivalent synapses onto principal cells. These experiments will shed light not only on the nature of synaptic plasticity of inhibitory interneurons but will help answer important questions remaining regarding presynaptic plasticity in the CA3 subfield.

3. Arachidonic acid regulation of transient potassium currents in principal and inhibitory interneurons.
We shall continue our experiments regarding the modulation of transient A-type currents in both CA1 pyramidal neurons and st. oriens/alveus interneurons. We will extend these studies to look at the biophysical mechanism of action of arachidonic acid block. In addition we will further characterize the nature of the interneurons sensitive and insensitive to arachidonic acid. In our preliminary experiments we have found a basket cell type resistant to arachidonic acid block, which is paradoxically regulated by extracellular potassium ion concentration. The physiological relevance of this regulation at this time is poorly understood. It is of extreme importance to our understanding of K channel modulation that the two classes of interneuron be identified. Such morphological identification will shed light on the nature of inhibition not only during physiological activity but during electrographic seizure activity.

4. Antisense knockout of Shaker family of potassium channels expressed on interneurons.
We will continue our studies on potassium channel function using antisense oligonucleotide targeted to knock specific K channel subunits in cultured hippocampal slices. Recent evidence suggests that Kv2.1 expression is upregulated by nerve growth factor (NGF) by an as yet unknown mechanism of action. We will extend our present studies to observe the effects of NGF treatment on K channel function in cultured hippocampal slices using both current and voltage clamp experiments. It is anticipated that these studies will reveal the nature of the role played by Kv2.1 in both principal cells and inhibitory interneurons of the mammalian hippocampus. In addition experiments are underway to determine the turnover rate of specific potassium channel subtypes in hippocampal neurons using pulse-chase experiments. The rate of synthesis and turnover will be studied in both control and NGF treated cultured hippocampus.

Significance to Biomedical Research and the Program of the Institute
Inhibitory interneurons represent ~10% of the total neuron population of the hippocampus proper and several distinct
The critical role inhibitory neurons play in the regulation of hippocampal excitability is underscored by the large number of existing seizure models which compromise the GABAergic inhibitory mechanism. Thus, an understanding of the basic

physiological and pathological properties of these neurons may elucidate mechanisms which could be useful for the management or the prevention of seizure activity in the hippocampus. To this end we have focused on the compliment of voltage gated potassium channels on these cells and their roles in various forms of synaptic plasticity. We anticipate that the characteristic physiological properties of these cells are due largely in part to the compliment of voltage gated channels present on these neurons. It is a long term goal of this lab to exploit the novel potassium channels present on these cells responsible for the regulation of their excitability.

Publications

Chikwendu A, McBain CJ. Two Temporally Overlapping "Delayed-Rectifiers Determine the Voltage-Dependent Potassium Current Phenotype in Cultured Hippocampal Neurons. *Journal of Neurophysiology 1996;76 3: in press.*

Du J, Zhang L,Weiser M, Rudy B, McBain CJ. .Developmental Expression and Functional Characterization of the Potassium Channel Subunit Kv3.1b in Parvalbumin-Containing Interneurons of the Rat Hippocampus. *Journal of Neuroscience 1996;16: 506 - 18.*

Gallo V, Zhou JM, McBain C J, Wright P, Knutson PL, Armstrong R C. Oligodendrocyte Progenitor Cell Proliferation and Lineage Progression are Regulated by Glutamate Receptor-Mediated K^+ Channel Block. *Journal of Neuroscience 1996; 16: 2659 - 70.*

Maccaferri G , McBain CJ. The Hyperpolarization-Activated Current (Ih) and its Contribution to Pacemaker Activity in Rat CA1 Hippocampal St. Oriens/Alveus Interneurons. *Journal of Physiology 1996; in press.*

Maccaferri G, McBain CJ. Long-Term Potentiation in Distinct Subtypes of Hippocampal Non-Pyramidal Cells. *Journal of Neuroscience 1996; 16: in press.*

McBain C J, Maccaferri G. Synaptic Plasticity in Hippocampal Interneurons?: A Commentary. *Canadian Journal of Pharmacology and Physiology 1996; in press.*

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01206 03 LCMN

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Receptor Mediated Calcium Signalling in Glia and Neurons

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	J.T. Russell	Head, Section on Neuronal Secretory Systems	LCMN, NICHD	12 months
Others:	L.A. Holtzclaw	Biologist	LCMN, NICHD	12 months
	N. Darvish	Visiting Fellow	LCMN, NICHD	10 months
	P.B. Simpson	Visiting Fellow	LCMN, NICHD	12 months
	D.B. Langley	Visiting Fellow	LCMN, NICHD	12 months

COOPERATING UNITS *(if any)*

Dr. V. Gallo, Head, Unit on Neurobiology, LCMN, NICHD, Dr. Alan Sharp, Department of Neurology, John Hopkins, Dr. D. Jacobowitz, Head, Sect. on Histo-Pharmacology, LCS, NIMH.

LAB/BRANCH

Laboratory of Cellular and Molecular Neurophysiology

SECTION

Section on Neuronal Secretory Systems

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	3.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This section aims to understand the principles and mechanisms that govern intracellular calcium signalling in glial cells and neurons. Although neurons are the central players in cellular communication and information processing in the brain, recent discoveries of glial cell types, excitability and signalling properties have shown that glial cells play an important role in long distace signaling in the brain. While glial cells lack electrically excitable properties similar to neurons they do, however, possess a form calcium based excitabilty in response to activation of neurotransmitter receptors. In glia, like in other cells, calcium signals propagate as waves over the entire cell including fine processes. The research focus of the Section includes studies on the signal transduction mechanisms of various receptor systems in glial cells as well as the cell biology of calcium signalling in both glia and neurons. One objective is to understand cellular processes that control signal transduction mechanisms for Ca^{2+} signalling and processes that support temporal and spatial signalling within cells and between cells at the molecular level. A second objective is to understnd the processes that regulate calcium based excitability in glial cells resulting from neuronal activity. Thirdly, to investigate the changes in the signalling modalities in reactive astrocytes during brain injury. Finally, this Section aims to describe in detail the ionic basis for cellular excitability in pineal cells.

Project Description:

Objectives: The aim of this Section is to describe in detail glial cell signalling in response to neuronal activity in the normal brain and in brain injury. During neuronal activity, it is believed that astrocytes monitor synaptic activity and respond to released transmitters with intracellular calcium signals by the activation of metabotropic and ionotropic receptor systems. These signals are propagated as waves within single astrocytes and are transmitted through a complex network of glial cells connected by gap junctions over long distances, albeit at rates considerably slower than (approximately 1000 times) neuronal signals. This type of long distance communication between glial cells has been termed calcium based excitability. A number of lines of work has clearly shown that glial cells *in situ* express functional receptor systems for neurotransmitters. Moreover, recently, glial cell calcium signals following neuronal stimulation have been recorded in Schwann cells in peripheral nerves. In another set of experiments on hippocampal slices in organotypic culture, neuronal stimulation resulted in glial calcium waves. The singular question that still remains unanswered is, if such signalling occurs in the intact brain during normal brain function. *This Section's efforts are focused on investigation of the cellular mechanisms of propagation of calcium waves within glial cells and their processes and in glial cell networks over long distances. Secondly, the Section also is investigating the membrane currents that form the basis for excitability in mammalian pineal cells.* We have characterized Ca^{2+} waves in glial cells in culture in some detail and have described a mechanism of saltatory propagation of these waves within cell body and processes. We have also shown that the saltatory nature of propagation results from local specializations in the Ca^{2+} release machinery. During the year 1996- '97, we aim to study the following aspects of signal transduction and glial cell calcium signalling: (i) Investigate in molecular detail the specializations in both the endoplasmic reticulum membrane system and organelles that support Ca^{2+} accumulation and release during wave propagation. (ii) Investigate mitochondrial involvement in the calcium release process in glial cells. (iii) Investigate the structure of endoplasmic reticulum in glia with reference to its Ca2+ signalling function. (iv) Investigate calcium based excitability in neuronal-glial networks *in situ.* (v) Characterize the ionic channels that support signal transduction of adrenergic and peptidergic receptors regulating melatonin synthesis and release in rat pineal cells..

Methods Employed:

Primary culture of glial cells isolated from neonatal rats, high resolution fluorescence microscopy, digital image processing, immunocytochemistry, Electrophoresis and Western blotting, electrophysiological recordings.

Major Findings:

A. Calcium signalling in glial cells of astrocyte and oligodendrocyte lineages..

1. Agonist-induced calcium waves

This Section has studied the qualitative and quantitative aspects of cytoplasmic calcium wave generation and propagation within single glial cells isolated from new born rat cerebral corticesand maintained in culture. Stimulation of glial cells with glutamate, norepinephrine or methacholine elicited intracellular calcium signals as waves that propagated through the cell. Our analysis has revealed that these agonist induced Ca^{2+} waves begin at a single cellular site and are propagated in a non-linear fashion with regenerative amplification at other specific cellular regions. High resolution analysis of the cellular loci in a number of experiments showed that the diameter of these regenerative loci ranged from 7 µm to 15 µm (12 ± 4 µm, mean ± S.D., n=28). Repeated application of an agonist always initiated Ca^{2+} waves at the same locus with an identical spatio-temporal oscillatory pattern.

In any given cell, the wave initiation locus and amplification sites remained invariant, We also found that the active loci have independent oscillatory properties and can oscillate at very different frequencies and possess different latencies. Nevertheless, these loci are influenced by diffusional waves reaching them from adjacent cellular regions and thus act as partially coupled oscillators. The local waves generated at the various cellular loci, when out of phase with each other, and are propagated in opposite directions, collide and annihilate. A mathematical model was developed which included spatial diffusion of Ca^{2+} in a cell with discrete active cellular loci of wave amplification. This model predicted that the intrinsic latencies of each locus are gives the appearance of wave behavior. Nearly simultaneous excitation of the entire cell at high agonist concentrations argues against the idea of a "wave" through an excitable medium, but rather suggests differences in the kinetic behaviour of the different oscillators,which is consistent with the experimental data.

The hypothesis that propagation of Ca^{2+} waves in glial cells and processes are supported by enhanced Ca^{2+} release at specialized cellular sites was developed mainly from experimental observations in type 1 and to a certain extent in type 2 astroglia. The investigation was extended to cells of the oligodendrocyte lineage because of their functional (in myelination) and structural (having long and slender cellular processes) specializations. Stimulation of oligodendrocytes with 100 µM MCh resulted in the activation of propagating Ca^{2+} wave fronts from several initiation sites . Although oligodendrocyte processes are approximately cylindrical in nature, the amplitude and rate of rise of the local Ca^{2+} signals were found to be non-uniform, with some regions of the processes displaying markedly higher amplitude responses and faster rate of rise of response, than in surrounding regions. These appear highly analogous to the amplification regions previously described for $InsP_3$-mediated responses in type 1 astrocytes, whose elevated Ca^{2+} kinetics probably contribute to the sustenance and propagation of the wave. In oligodendrocytes the specialized regions of wave initiation and amplification varied in diameter from 1-5 µm.

Our investigation of mechanism of propagation of Ca^{2+} waves in glial cells and their processes resulted in the following functional description of the Ca^{2+} release process: In glial cells, the functional intracellular Ca^{2+} release pool is organized into discrete functional units, which give rise to highly localized Ca^{2+} signals in cellular sites upon activation. These units are spread throughout the cells and can either operate independently of be coupled by diffusion of Ca^{2+} from one release site to another to generate a Ca^{2+} wave depending on the stinulus. The exact physical nature of the Ca^{2+} release sites is unclear. This hypothesis, however, is supported by the observation that wave initiation and amplification sites remain invariant during repeated stimulations by the same agonist or a different agonist. In addition, Ca^{2+} waves initiated by pharmacological activation of ryanodine receptor channels or by thapsigargin which inactivates the endoplasmic reticulum SERCA pumps also show similar spatio temporal kinetics. These experimental observations lead us to conclude that the cellular sites of enhanced Ca^{2+} release might be due to specializations in multiple cellular processes involved in Ca^{2+} homeostasis and signalling. With this realization we systematically studied the distribution and functional characteristics of $InsP_3$ receptors ($InsP_3Rs$), ryanodine receptors (RyR), SERCA Ca^{2+} pumps and distribution of endoplasmic reticulum membranes, its luminal Ca^{2+} binding proteins and mitochondria in glial cells and their processes.

2. Distribution of $InsP_3Rs$ in glial cells.

We initially hypothesized that the regions of specialized Ca^{2+} release in glia may be due to high density distribution of $IInsP_3Rs$ on the endoplasmic reticulum in these cells. $InsP_3R$ was localized by immunocytochemistry and a manuscript describing this work by Dr. Carol Sheppard is currently under revision.

We obtained a number of different antibodies against the different types of $InsP_3R$ from collaborating laboratories for immunocytochemical analysis. We asked the following questions: (1) What is the $InsP_3R$ distribution in the ER of tissue cultured astrocytes? (2) Does this distribution support the kinetic pattern of wave propagation measured in the same cell? (3) Are the different types of $InsP_3Rs$ expressed in the same cells, and if so do they show different InsP3 sensitivities in different regions of the cell? In addition, antibodies against specific cell identification markers such as glial fibrillary acidic protein (GFAP) and ganglioside-3 (GD-3) and galactocerebroside-C (Gal-C) were used to positively identify glial cell types.

Endoplasmic reticulum in cultured glial cells was decorated by antibodies against all three types of $InsP_3R$ ie. type 1 $InsP_3R$, $InsP_3R1$; type-2 $InsP_3R$, $InsP_3R2$; and type-3 $InsP_3R$, $InsP_3R3$. Dual labelling with cell type specific antibodies and $InsP_3R$ selective antibodies clearly showed that all three subtypes of receptor were found in each cell. The overall distribution pattern of these receptor subtypes, however, was different in any given cell (see later). In general the receptor distribution was punctate with occasional clustering and the size of the clusters was not uniform. In some regions of the cell, relatively large clusters of receptors were found compared with other regions.

Distribution of the three subtypes of receptors differed significantly from each other with each subtype showing unique subcellular distribution patterns. Antibodies specific to InsP$_3$R1 intensely stained the perinuclear ER membranes and the nuclear envelope. Similarly, intense staining for IP$_3$R3 was also found in the perinuclear region while in the processes the staining was considerably less compared with IP$_3$R2. The perinuclear distribution of the two receptor subtypes was confirmed by high resolution analysis using digital confocal microscopy. In order to verify that the staining is restricted to the perinuclear membrane and did not extend into the nuclear matrix, the extent of staining in the Z-dimension was measured for a number of receptor complexes and compared with the axial resolution achieved by the microscopic technique. The axial resolution of the fluorescence microscope using the digital confocal algorithm was calibrated using fluorescently labeled latex spheres of known diameter (200 nm) filled with fluorophore. Analysis of the pixel intensity profiles of a number of such beads (Fig. 7*D*) showed that the axial resolution, measured as full width at half maximal intensity, for the microscope was 0.448 ± 0.028 µm (Mean ±SEM, n=14). Similar measurements were made in 12 different receptor complexes and showed that the fluorescence intensity (full width at half maximal intensity) extended 0.426 ± 0.028 µm (Mean ±SEM), suggesting that the stained receptor complexes are smaller than the resolution limit of the microscope. Together with the qualitative analysis using 3D voxel rendering, this quantitative measure showed that the staining for IP$_3$R1 receptor complexes is present on the perinuclear membranes and did not extend to the nuclear matrix (9 separate cells). Examination of IP$_3$R3 distribution in this manner also showed similar expression in the nuclear envelope (6 separate cells). Whether the receptors are present in the inner or the outer nuclear membrane is not known. IP$_3$R2 staining was present throughout the cell, was punctate and extended even through fine processes. Staining in the nuclear membrane and the perinuclear region was not particularly different from other parts of the cell.

3. Comparison of the patterns of IP$_3$R distribution and Ca^{2+} release

Growing astrocytes on glass cover slips etched with a grid pattern enabled us to measure agonist-evoked Ca^{2+} signals in living cells, and to subsequently identify and perform immunocytochemistry on the same cells. We compared the distribution of IP$_3$R2 with the wave propagation kinetics, in astrocytes and oligodendrocytes. Upon stimulation with norepinephrine (500 nM), type 1 astrocytes responded with an increase in intracellular Ca^{2+} that propagated as a wave through the cell. Wave propagation was analyzed in offset plots fluo-3 fluorescence intensity values measured through time in a series of adjacent slices through the cell image. The local kinetics of Ca^{2+} release was measured in the individual traces to obtain peak amplitudes and rates of rise of the response. Staining with anti-GFAP antibody provided positive identification of the cell as an astrocyte. The quantitative analyses of the kinetic parameters of the Ca^{2+} wave was compared with the distribution of IP$_3$R2 immunoreactivity of in the same celsl. The peak amplitude of Ca^{2+} and the maximum slope of Ca^{2+} release along the cell showed similar patterns. Qualitative comparison showed that the high density InsP$_3$R2 staining observed in some regions of the cell correlated with regions of elevated Ca^{2+} release kinetics.

In order to make a quantitative comparison between InsP$_3$R2 distribution and wave propagation kinetics, the cells were imaged using the same intensified CCD camera in the fluorescence microscope following immunocytochemical staining and the fluorescence intensity was measured in the series of slices where the Ca^{2+} release kinetics were previously measured in the same cells. The pattern of staining was compared with the pattern of maximum slope of Ca^{2+} release and the amplitudes of the local signals. By and large, the pattern of receptor distribution closely matched the local wave kinetics. A quantitative comparison between the spatial distribution of the kinetics of Ca^{2+} release and IP$_3$R2 distribution was carried out using a cross correlation analysis. Cross correlation is the inverse Fourier transform of the cross-spectrum, and is calculated by taking two Fast Fourier Transforms (FFT) of the data sets to be compared. Cross-spectra were then formed as a product of the transform of one wave with the complex conjugate of the transform of a second wave. Inverse Fourier transformation of the cross spectrum produced the cross-correlation function. The cross correlation function between IP$_3$R2 distribution and local slopes in Ca^{2+} release and local amplitudes gave values greater than 0.9 and centered very near zero. This indicates that the shape of these two measured patterns are very nearly the same. In all, comparison of wave kinetics and IP$_3$R2 distribution were carried out in 12 individual cells and in three of these experiments quantitative comparison using cross correlation analysis was carried out which showed cross correlation functions higher than 0.7. The cross correlation peak was near zero indicating that the two distributions are not only similar but are spatially colocalized. Changing the order of comparisons in every case produced the expected mirror image cross correlation functions at identical levels. This analysis showed that IP$_3$R2 distribution in cells correlated well with areas where Ca^{2+} release kinetics were elevated suggesting that high density receptor distribution is at least one mechanism by which the cells achieved high local Ca^{2+} release.

4. Distribution of ryanodine receptors (RyRs) in glial cells.

Immunocytochemical analysis showed that the cell bodies of glial cells were heavily stained with RyR specific antibodies. Lesser, variegated expression was present in the distal processes. Parallel experiments with type 2 astrocytes confirmed our previous finding that significant RyR expression is also present in these glial cells. The expression patterns of RyRs in oligodendrocytes, O-2A cells and type 2 astrocytes appear to bear broad similarities, with staining in each being highest in the cell body, and expression in processes appearing punctate and variegated. It should be noted that RyR expression is evident along even very thin glial cell processes, with particularly high levels in some areas of these processes compared to surrounding regions giving a bead-like appearance.

To examine the distribution in higher resolution, we used the digital confocal restoration technique. The results of this analysis showed that within the cell body region of O-2A progenitor cells, RyR expression is apparent everywhere except within the nucleus. Several large conglomerations were apparent, however, particularly in the perinuclear region. The perinuclear distribution can be clearly appreciated in 3D reconstruction of confocal images and rendering them in stereo pairs. In oligodendrocytes, expression in the cell body was also found to be widespread, but much more marked around the nucleus. Similarly, type 2 astrocytes were found to express RyRs in a variegated but widespread manner, with expression being highest on a membrane-like structure apparently encircling the nucleus. Not all areas of the cell body of type 2 astrocytes appeared to express detectable levels of RyRs. In all the glial cell types examined, RyR expression thus appears to be most prominent on specialized regions in these cells which may be analogous to a perinuclear membrane apparatus found to possess a high level of sarcoplasmic-endoplasmic reticulum Ca^{2+}ATPase in type 1 and type 2 astrocytes. It appears possible that RyRs are expressed in the nuclear envelope in these cells together with $InsP_3$ receptors.

Functional studies using Ca^{2+} measurements showed that caffeine (50 mM) which opens ryanodine receptor channels, caused a Ca^{2+} response in most type 2 astrocytes and in a high percentage of oligodendrocytes, but had little effect on O-2A progenitor cells. Caffeine responses were inhibited by pretreatment with ryanodine (10 μM) or thapsigargin (1 μM), and the peak response was unaffected by removal of $[Ca^{2+}]_o$. These findings suggested that the caffeince induced Ca^{2+} signals are due to release from ER stores and that, in glial cells, ryanodine receptors expressed on ER membranes are functional. When analyzed with high spatial and temporal resolution, caffeine responses in both type 2 astrocytes and oligodendrocytes were found to be waves propagating in a non-linear manner from several initiation sites via multiple amplification zones similar to agonist-induced waves. Initiation and amplification sites were reproducible for successive caffeine stimulations in a given cell, consistent with them corresponding to distinct cellular specializations. Pretreatment with kainate (200 μM) increased the magnitude of caffeine-evoked $[Ca^{2+}]_i$ elevations in type 2 astrocytes, and caused caffeine to activate responses in a significant proportion of previously non-responding O-2A progenitors. Our results demonstrate that cells of the O-2A lineage possess functional ryanodine receptors, expressed on Ca^{2+} stores which appear to be refilled by kainate-activated Ca^{2+} entry. Activation of these receptors evokes Ca^{2+} waves whose magnitude and rate of propagation varies throughout the glial arborisation in a characteristic manner. Ryanodine receptors could thus play an important role in glial cell signaling.

It was important to determine that the expression of ryanodine receptors is not a peculiarity of glial cells in tissue culture. In order to examine glial cells *in situ*, we carried out immunocytochemical staining of sections of rat brains which have been perfusion fixed with formaldehyde. Dual immunohistochemical staining using antibodies against glial cell markers (Gal-c and GFAP) and anti-RYR antibodies conclusively proved that both astrocytes and oligodendrocytes express ryanodine receptors *in situ*.

5. Comparison between $InsP_3$- and caffeine-evoked Ca^{2+} waves

Our experiments showed that cells of the oligodendrocyte lineage respond to caffeine with propagating Ca^{2+} waves whose local kinetics vary along the length of the cell. We then compared, in the same cells, $InsP_3$-mediated (MCh-induced) and caffeine-mediated waves. These experiments showed that the characteristics of the Ca^{2+} waves were similar under both stimulation conditions since the local amplitudes of the signal and rates of rise were similar in the two responses. These experiments showed that the specialized regions of high amplitude Ca^{2+} release sites were identical for InsP3 dependent waves as well as RyR dependent Ca^{2+} waves. Cross correlation analysis of the caffeine peak amplitude *vs* MCh peak amplitude showed high correlation values in phase. In three separate experiments, the cross-correlation values were 0.70 ± 0.05 (mean ± SD). The pattern of cross correlation is similar to the autocorrelation of the local peak amplitude data for the caffeine response. Comparison of the rates of release of Ca^{2+} (slopes) between the two responses also yielded similar high cross correlation values (0.82 ± 0.17; n=3). This result indicates that a local cellular specialization that supports high Ca^{2+} release may be due not only to increased expression of $InsP_3R$, as suggested by us previously, but to a generalized specialization of the Ca^{2+} release machinery, perhaps including Ca^{2+}-induced Ca^{2+}-release processes and specialization of other organelles (see later).

6. The unitary Ca^{2+} release machinery

Over the last three years, our laboratory has shown that in glial cells, propagation of Ca^{2+} waves is supported by cellular specializations that support highly localized Ca^{2+} signals. It has become clear that these specializations may include coordinate localization of multiple cellular elements involved in Ca^{2+} homeostasis and signalling. It is likely, that glial cells organize their cytoatrchtecture such that Ca^{2+} release sites with enhanced kinetics are placed depending upon the requirements for both intracellular and intercellular signalling. We investigated the nature of these structural and functional specializations using the strategy we developed to compare IP_3R distribution with Ca^{2+} release kinetics. We measured Ca^{2+} waves in cells and then fixed them with gluteraldehyde and examined their endoplasmic reticulum structure, its luminal contents and, recently, distribution of mitochondria. These studies included investigation of the distribution of SERCA2 Ca^{2+} pumps, and the major luminal Ca^{2+} binding protein in nonmuscle cells, calreticulin by immunocytochemistry. In every case, the specificity of the antibodies was verified by polyacrylamide gel electrophoresis of glial cell extracts followed by Western blotting. The presence of the specific antigen was also verified in intact tissue by immunohistochemistry of brain sections. In addition, we used $DiOC_6(3)$, JC-1 and mitotracker to decorate the ER membranes and mitochondria.

(I). Endoplasmic reticulum specializations.

The endoplasmic reticulum was stained using $DiOC_6(3)$ a carbocyanine dye that intercalates within the bilayer in most membranes. When the endoplasmic reticulum was stained with Di-O-C6-3, the ER membrane system was found to extend over the entire cell, including small cellular processes. This finding would suggest that, like in other cells, in astrocytes the ER is a continuous cistern and participates in calcium signalling. The distribution of IP_3R2, which is found throughout the cell, closely matched this $DiOC_6(3)$ staining pattern in all cells showing that IP_3R2 is found everywhere. Since $DiOC_6(3)$ is a non-specific membrane dye, it stained, in addition, mitochondria, which appeared rod like in both the cell body and processes. Together with variations in ER membrane density, the mitochondrial staining by $DiOC_6(3)$ made the glial cell processes appear like a string of beads with periodic intensely staining spots.

(a) SERCA type Ca^{2+} pumps on endoplasmic reticulum in glial cells

An antibody against SERCA2 was used to visualize the subcellular distribution of the ER calcium-sequestering pumps. The antibody has been shown to recognize a single 100 kDa protein band in immunoblots of extracts from human neutrophils. Examination of astrocytes stained with this antibody revealed that the ER was diffusely stained in both type 1 and type 2 astrocytes. In addition, the presence of an intensely stained substantial perinuclear membrane network was evident in both cell types. This perinuclear apparatus appeared as a highly interconnected cistern-like structure and was by far the most heavily stained region of the cell. While the SERCA2 pumps seem to be distributed over the entire ER membrane system, the distribution appears spatially heterogeneous, with some regions of the cell showing much higher density of pumps than others. In addition to immunocytochemistry, the SERCA pumps were directly visualized by labelling with fluorescent thapsigargin which also yielded similar results.

(b) Calreticulin distribution.

Calreticululin distribution in astrocytes and oligodendrocytes was examined by immunocytochemistry. This calcium binding protein has been identified as the major ER Ca^{2+} binding protein in non-muscle cells. In glial cells, calreticulin was distributed in a highly heterogeneous manner and appeared concentrated in some areas of the endoplasmic reticulum. Because of this heterogeneous distribution, the glial cell processes appeared like a chan of beads, the beads being concentrations of calreticulin. We compared the local Ca^{2+} release kinetics during propagation of agonist stimulated Ca^{2+} waves with the distribution of calreticulin in the same cells. Long cellular processes were chosen for this study since these processes can easily be treated as two dimensional objects. Quantitative cross correlation analysis showed that locally high Ca^{2+} release sites were highly correlated with the sites where calreticulin was concentrated. Cross correlation values of 0.8 to 0.9 were obtained. This result suggested that in glial cell processes calcreticulin is specifically concentrated in areas where elevated Ca^{2+} release kinetics exist.

(ii) Mitochondria

The carbocyanine dye, $DiOC_6(3)$ stained both the endoplasmic reticulum and mitochondria which appeared rod shaped in cell bodies as well as processes. Our inital analysis of Ca^{2+} waves and local Ca^{2+} release kinetics showed that the rod shaped $DiOC_6(3)$ stained bodies always were associated with the regions of high Ca^{2+} release kinetics. This finding prompted us to investigate in detail the relationship of mitochondrial distribution and function to Ca^{2+} signalling and Ca^{2+} wave propagation. Other cyanine dyes more specific to mitochondria were used to localize the organelle and to compare their distribution with local kinetics of Ca^{2+} release. We used $DiOC_6(3)$, JC-1 and mitotracker as organelle specific dyes. JC-1, the carbocyanine dye, partitions into mitochondrial membranes and at low membrane potential emits at 527 nm when excited at 490 nm. At high membrane potentials, JC-1 forms J-aggregates and the emission wavelength shifts towards red (590 nm), enabling the dye to function as a highly specific marker both of mitochondrial location and activity. A third mitochondria specific dye, mitotracker was used, ina ddition, to specifically label this organelle. Mitotracker is extremely brightly fluorescent and highly specific to mitochondrial membranes and allowed for fixation and immunocytochemical staining for other antigens for colocalization studies. In addition, mitotracker was used to monitor organellar motility in living cells.

(a) Comparison Ca^{2+} release kinetics with distribution of mitochondria.

All three dyes showed that mitochondria are found at highest density in the cell body region, but were also present along cellular processes. In thicker processes, groups of three or more mitochondria can be typically found in a convoluted, closely interrelated group, at a considerable distance from the next such group or individual mitochondrion, while in thin processes, single, long mitochondria are found with several µm-separations. This is similar to mitochondrial distribution described in cultured neurons, but in several other types of cells in culture, mitochondria are not found along processes. While bright $DiOC_6(3)$ staining marked groups of mitochondria along processes, less intense $DiOC_6(3)$ staining is diffusely distributed through the cell, indicating that ER is present throughout the network of oligodendrocyte processes. With JC-1, all mitochondria fluoresce green, while parts of most of them also were red, indicating variations in membrane potential (and corresponding level of activity) within single mitochondria, and between different mitochondria in a given cell. The finding of single or groups of mitochondria along oligodendrocyte processes, filling most of the available space and apparently in close proximity to ER, indicates that the potential exists for functional interactions between ER and mitochondria in these cells.

(b) Functional importance of mitochondria in Ca^{2+} signalling

To investigate whether the distribution of mitochondria is related to characteristics of the Ca^{2+} waves evoked by store release, we studied Ca^{2+} waves induced by MCh (100 µM), and compared the spatial kinetics with the mitochondrial distribution in the same cells. If mitochondrial function is fundamental to Ca^{2+} signaling in cellular microdomains, (i) the pattern of mitochondrial distribution will be expected to be well correlated with the kinetics of local Ca^{2+} release and (ii) altering mitochondrial function would alter local kinetics in Ca^{2+} signaling. To test these hypotheses, we localized mitochondria in cells where the kinetics of Ca^{2+} release were measured and studied the effect of mitochondrial inhibition on these kinetics. Ca^{2+} waves were evoked by MCh in fluo-3 loaded cells and, following recovery, they were incubated with JC-1 or $DiOC_6(3)$ while still on the microscope stage. The fluorescence of this second dye was measured, and the resultant wave and organellar expression patterns were then compared. This analysis showed that peaks in the Ca^{2+} response were found at sites along processes only where mitochondria were present, and mitochondria were not found at sites where response peaks were not observed. The spatial pattern of Ca^{2+} release kinetics was compared with the pattern of distribution of mitochondria in glial cell processes using cross correlation analysis. Using this analysis the local amplitudes and the rate of rise of the Ca^{2+} signal was compared with the patterns of JC-1 or $DiOC_6(3)$ florescence. Comparison of the patterns of $DiOC_6$ (3) staining intensity along the cellular process (distribution of mitochondria) with the patterns of both the amplitude and rate of rise of the Ca^{2+} response showed high correlation values approximately 2-3 µm from phase. The local amplitudes and the rates of rise (slope) of the Ca^{2+} responses when compared with each other showed good correlation values in phase. With JC-1, the magnitude and the pattern of cross correlation was similar between wave characteristics and either JC-1 green fluorescence or red J aggregates. This result may suggest that the location of mitochondria rather than its local level of activity may be most crucial in determining wave characteristics in oligodendrocytes.

To investigate the nature of the role played by mitochondria in Ca^{2+} signaling in oligodendrocytes, the effect of modifying mitochondrial activity on MCh-evoked Ca^{2+} signals was studied. After initial stimulation with MCh, cells were treated with the protonophore FCCP (1-5 µM) which collapses the proton gradient across mitochondrial membranes, resulting in inhibition of Ca^{2+} uptake and depletion of Ca^{2+} from mitochondria. FCCP (5 µM, 30 min) pretreatment greatly reduced or abolished the MCh-evoked Ca^{2+} response. Relatively brief (2-5 min) pretreatment with 1 µM FCCP caused MCh responses in some cells to be abolished, but responses in other cells to be markedly potentiated, while others were unaffected. FCCP treatment by itself evokes a slow, persistent elevation of $[Ca^{2+}]_i$ in oligodendrocytes, probably due to leak of Ca^{2+} out of mitochondria. In most cells examined, a 2 min pretreatment with CCCP (5 µM), a structural analog of FCCP, completely abolished MCh-induced $[Ca^{2+}]_i$ signals). An alternative approach to modulation of mitochondrial function was treatment of the cells with antimycin, an inhibitor of electron transport through complex III . Incubation with antimycin (2 µg/ml) for as little as 2 min inhibited Ca^{2+} responses to MCh stimulation in most cells, although by itself antimycin has only a minimal effect on basal $[Ca^{2+}]_i$ levels. Prolonged treatment with antimycin had no greater effect on Ca^{2+} signals, while a lower concentration (0.2 µg/ml, 15 min) also inhibited MCh-evoked responses to a marked extent. The results of this study demonstrate that propagation of Ca^{2+} waves in glial cell processes dependent on mitochondrial location and function.

The focus of the Section has been to understand in detail the mechanisms of Ca^{2+} wave propagation in glial cell processes over long distances. We were particularly interested in the nature of the unitary Ca^{2+} release machinery. Our studies show that the enhanced Ca^{2+} release sites are endowed with 1. high density $InsP_3Rs$, high density RyRs, higher concentration of calreticulin within the ER lumen and, in addition, a collection of or at lease one mitochondrion. In this way the cell assembles a specialized collection proteins and organelles to set up a Ca^{2+} release machinery which can support wave propagation and local Ca^{2+} signals no matter what the original trigger may be ie. metabotropic receptor-mediated signals or RyR mediated signals.

B. Pineal cell calcium signals

A second focus of the Section is to study circadian regulation of ineal cell excitability by neural control. Nissim Darvish, a visiting fellow established a patch clamp rig in the Section. This has been a much anticipated addition to the laboratory, since it allows for exploring ionic channel mechanisms involved in calcium signalling. Although this approach was initially undertaken to characterize the cyclic GMP gated cation channel in pineal cells, the methodology provides a new dimension in our analysis of glial neuronal interactions as well. This system is currently fully operational and Dr. Darvish has completed a series of studies using the system.

1. PACAP receptor mediated calcium signals in pineal cells

PACAP (Pituitary Adenylyl Cyclase Activating Peptide) is a 38 amino acid peptide which shares considerable sequence homology with VIP and is found in nerve fibres innervating rodent pineal. Since our previous studies showed that the signal transduction of VIP receptor may utilize the cyclic GMP-gated cation channels, we wanted to examine if PACAP also stimulated this receptor complex. Preliminary experiments showed that PACAP at a concentration of 0.1 µM caused an increase in intracellular Ca^{2+} in isolated rat pineal cells. The pattern of these signals, however, showed a peak followed by plateau type of response. This pattern is distinct from that seen when VIP receptors are stimulated, which produced a transient peak response. In addition, unlike the VIP stimulated calcium signals, the PACAP response persisted in the absence of extracellular Ca^{2+}. Furthermore, depletion of cellular calcium stores using thapsigargin (0.5 µM), an inhibitor of ER calcium pumps, abolished the PACAP response. These observations suggested that the PACAP response is dependent upon release of calcium from intracellular stores. The signal transduction mechanisms that underlie this PACAP-induced calcium release from pineal cell ER are under investigation.

The patch clamp studies on pineal cells utilized various different configurations, namely, on-cell, whole-cell, perforated patch and isolated patches. By the use of these approaches the following ionic conductances have been identified in pineal cells. These included a nonspecific cation channel, the cGMP gated cation channel, an inwardly directed K^+ channel and an outwardly directed K^+ channel. We decided to focus on the nonspecific cation channel, since it was modulated by the major neruotransmitter that controls pineal function.

2. Novel mechanism of modulation of a nonselective cation channel in the pineal .

In rats, melatonin synthesis and secretion increases in the evening and is maintained elevated over several hours during the night. The cellular mechanisms by which long lasting increase in both $[Ca^{2+}]_i$ and cAMP levels upon activation of adrenergic receptors by norepinephrine released by nerve endings of the superior cervical ganglionic innervation of the pineal. The mechanisms by which N-acetyl transferase expression is regulated are not known. In many sensory systems, cyclic nucleotides act through non-selective cation channels (NSCC) in the transduction of sensory stimuli and different types of NSCC expressed in different cell types, are central to the control of membrane potential and thus excitability. In avian pineal cells, a NSCC controlled by the intrinsic circadian clock has recently been identified. In this study, we investigated membrane currents modulated by the two major regulatory neurotransmitters that control melatonin secretion by the rat pineal, namely, NE and PACAP and have identified a nonselctive cation channel expressed in high density on these cells. This channel shows a novel form of cAMP dependent modulation such that NE, PACAP and 8BrcAMP, all (1) activate the channel to increase P_o; (2) upon prolonged stimulation, cause reduction of unitary conduictance and; (3) changes the rectification properties such that the channel carries inward currents.

NE, PACAP and 8BrcAMP increase channel P_o by shifting the voltage dependence to the right such that channel activity could be measured even under hyperpolarized membrane potentials. The slope of the exponential fit to the voltage dependence curves were not significantly different before and after treatment with agonist, suggesting that there was no change in the intrinsic voltage sensitivity of the channel. In addition to the increase in channel P_o, prolonged treatment results in a large decrease of unitary conductance measured in single channel recordings and whole cell currents. The molecular mechanisms that underlie this phenomenon remains largely unknown and sevaral possibilities exist. Our experiments argue that this decrease in unitary conductance may be due to intracellular accumulation of a blocker with time after agonist exposure which causes a fast channel block.

Prolonged receptor activation results in the conversion of the normally outwardly directed conductance to pass inward currents through the same channel. The following observations argue that the outward and inward currents are measured through the same channel complex. (1) The I/V relationships of the outward and inward currents measured through the NSCC were fitted with a simple linear regression (r=>0.9), suggesting that the unitary conductance and the ion selectivity are identical under both circumstances. (2) The voltage sensitivity was not different between the outwardly directed openings and the inward currents. (3) Inward currents appeared in 13 out of 21 trials. It could be argued that cAMP accumulation upon receptor activation increases channel P_o by shifting the voltage dependence such that channel gating could now be observed under hyperpolarized voltage commands. No correlation exists, however, between the increase in channel P_o and the appearance of inward current: In several experiments, even though channel P_o increased dramaticaly we did not observe any inward currents, while in others, small increases in channel P_o was associated with appearance of inward currents. If a different dormant channel is present in the same patch carrying inward current, this second channel should have the same conductance, ion selectivity and voltage dependence and most of the time be expressed together with the outward channel on the membrane. Such a possibility is highly unlikely.

We interpret this data to mean that NE and PACAP acting through their common second messenger, cAMP causes a change in the rectification characteristics of the current through this NSCC. This result strongly argues for channel modulation by cAMP accumulation which results in a change of the channel protein or related proteins enabling inward currents through the channel pore. One hypothesis is that a cAMP mediated change in the channel complex reduces the affinity of the channel to the intracellular component which then dissociates. Since RpcAMPs did not mimic the effects of cAMP, it is likely that a cAMP dependent phosphorylation step is important for this change to occur. This novel mode of second messenger mediated channel modulation of an ion channel in the vertebrate to our knowledge has not been observed before.

Under resting membrane potentials (-35 to -50 mV) the NSCC of rat pineal cells will remain closed most of the time. Upon noradrenergic or PACAP activation, however, the channel will open at these membrane potentials contributing to depolarization of the cell, since under physiological conditions Na^+ is expected to be the major current carrier. Indeed, under current clamp conditions, we found that PACAP induces a slow onset, long lasting depolarization of about 12 mV which increases Ca^{2+} influx across the plasma membrane. Since pineal cells maintain a circadian rhythm in plasma levels of melatonin in rats, their activation is unique and lasts many hours each evening (4PM to midnight;). NE outflow from the superior cervical ganglion and PACAP act via cAMP and Ca^{2+}. Both second messengers are required for maximal activation of N-acetyl transferase activity to increase melatonin synthesis. Since Ca^{2+} release from the endoplasmic reticulum stores is finite, a long lasting depolarization could provide the mechanism for refilling the Ca^{2+} stores by gating voltage gated Ca^{2+} channels found in these cells and activation of NSCC by cAMP could support such a mechanism. Indeed, 8BrcAMP and stimulation of cAMP accumulation by forskolin treatment resulted in calcium influx in these cells. In summary, we describe here a nonselective cation channel in rat pineal cells which is modulated by neurotransmitter receptors through a cAMP dependent mechanism. The modulation could result in activation of the channel under resting membrane potentials when it normally is inactive and thus support Ca^{2+} entry for long lasting refilling of cellular Ca^{2+} stores..This work is currently under preparation for publication.

Significance to Biomedical Research and the Program of the Institute:

The experimental system is directed towards the understanding of the cellular basis for glial cell signalling mechanisms, and neuronal and glial cell interactions. Glial cell derived trophic factors are essential in neural development and maintenance of the cytoarchitecture. Understanding of the nature of the glial-neuronal interactions is essential to understanding the development of cellular organization in the central nervous system and the basis for glial mechanisms in neurodegenerative diseases. The methods developed is expected to provide fundamental insights into the functional and structural organization of calcium signalling processes in cells. In addition the work elucidates novel generalizable mechanisms of receptor mediated signal transduction.

Proposed Course:

A. Calcium signalling in glial cells

This year we aim to set up paradigms to study calcium based excitability in glial cells in response to neuronal activity. We propose to concentrate on two separate avenues: namely (1) characterization of the molecular mechanisms of Ca^{2+} signalling in glial cells and (2) measurement of glial cell signals in response to neuronal activityin the brain slice preparation.

1. Cell biology of Ca^{2+} signalling in glial cells (Simpson, Russell)

Work in this Section has developed the idea that in glial cells the intracellular Ca^{2+} pools functions as discrete units that give rise to highly localized cellular Ca^{2+} signals upon activation. These units are spread throughout cells and can operate independently of be coupled by diffusion of Ca^{2+} from one release site to another to generate a Ca^{2+} wave. The physical nature of the Ca^{2+} release sites is unclear. In order to understand both the nature of the local Ca^{2+} release sites and their mechanistic and kinetic parameters, we have used a paradigm combining measurement of intracellular Ca^{2+} signals with immunocytochemical or other forms of morphological characterization. Using this paradigm, we have shown that, in glial cells, local sites of enhanced Ca^{2+} release are endowed with higher density distribution of $InsP_3Rs$, high concentrations of calreticulin in the local endoplasmic reticulum and conglomerations of mitochondria. It appears also that the functional integrity of mitochondria is essential for the local sites to be fully operational. The precise molecular mechanisms by which Ca^{2+} release kinetics through $InsP_3Rs$ is influenced by calreticulin in the ER lumen and the proximity of mitochondria is not known. We plan to investigate this question by altering mitochondrial function. by the use of chemicals that influence the proton gradient, the cytochrome system involved in electron transport and with mitochondrial substrates that alter energetics.

The effect of such modification on cellular local Ca^{2+} signals will be investigated. The experimental paradigm will consist of measuring agonist-induced Ca^{2+} waves in cells before and after modification of mitochondrial function and making quantitative comparison of local wave kientics between the two conditions.

Although mitochondria have been found in close apposition with endoplasmic reticulum membranes, there is no direct evidence that mitochondrial membranes interact directly with proteins in the ER. We plan to address this question by the use of chemical cross linking agents. Photo activatable cell permeable protein cross linking agents will be used identify nearest neighbour proteins. Particularly, after cross linking antibodies against $InsP_3Rs$ and mitochondrial uniporter will be used to ask if these proteins are found in close proximity with each other. Similar strategy will be utilized to investigate other proteins (eg. VDAC) found on mitochondrial membranes.

In collaboration with Dr. David Jacobowitz, a molecular biological approach will be attempted. In these studies, DNA encoding calreticulin, calbindin, and calretinin will be transfected into the oligodendrocyte lineage cell line, CG-4 to examine if the wave propagation kinetics are altered by overexpression of these ER luminal and cytoplasmic calcium binding proteins. In addition, the effect of overexpression of the mitochondrial proteins such as bcl-2, and the uniporter on wave propagation and local Ca^{2+} release kinetics will be investigated.

2. Glial cell signalling *in situ*

Only one set of published experimental results exist in the literature showing in organotypic cultures of hippocampal slices glial cell signals in response to neuronal stimulation. All other experiments showing calcium based excitability in glial cells have been carried out in pure glial cell cultures or in mixed cultures of glia and neurons. Glial cells in culture continue dividing unlike cells *in situ* and express antigens such as vimentin and nestin which are the hallmarks of "reactive astrocytes". In addition, the effects of unidentified growth factors and trophic substances found in fetal bovine serum on glial cells, their receptor expression and signal transduction are uncontrollable under experimental conditions. Because of this reason, experimental models need to be developed to study glial cell excitability *in situ*. Recent studies from several groups have shown that glial cells *in situ* possess functional receptor systems for neurotransmitters and respond to exogenously applied agonists. No direct measurements of glial cell signals in response to neuronal activity are yet available. We plan to use the acutely isolated hippocampal slice preparation to study glial cell signals in response to neuronal activity.
(i)Studies on brain slices (Russell, Simpson, McBain)

The brain slice preparation will be used to study glial cells *in situ* using the Noran confocal microscope. To begin with, the experiments planned will take the course of previously published work on organotypic cultures of rat hippocampal slices. The goal of the studies on brain slices is to find answers to a number of questions: (1) does neuronal stimulation result in glial cell responses in nerve terminal fields in the intact brain slice? (2) How far are these signals propagated? (3) Are there special glial cell phenotypes involved in this form of glial signalling in the brain? (4) are the gap junctional coupling between glia modulated depending upon neuronal activity? (5) What is the status of coupling between neurones and glial cells?

This project will be initiated when the confocal microscope is ready and its initial calibration experiments have been completed. We expect this to be completed by November 1996 at the latest. The instrument is currently in place and we are in the process of setting up the perfusion assembly and electrophysiology rig associated with it. The transmitted light illumination also needs to be completed. When these improvements are in place, final optical and electronic calibrations will be completed. We have begun the processes of recruiting a postdoctoral fellow with experience in brain slice methodology. The project would be begun when the new fellow arrives in collaboration with Dr. Chris McBain (LCMN) and a number of experiments are planned. We are currently in communication with three different potential Fellows and one of them will join the group latest by Spring of 1997.

To investigate the initial question of whether neuronal activity results in glial cell signals we need to establish methods to monitor $[Ca^{2+}]_i$ in glial cells in a brain slice. We intend to use hippocampal slices because of the anatomical uniqueness and because the preparation is well established. We intend to take advantage of Dr. Chris McBain's extensive experience in the slice technique. A number of difficulties are expected, including satisfactory loading of glial cells with reporter dyes. Since reporter dye loading using acetoxymethyl ester forms is expected to degrade signals due to light scattering during optical measurements, we intend developing techniques to inject cells with dyes. Stimulating electrodes will be placed in the region of the dentate gyrus to stimulate mossy fiber tracts and glial cells in the CA3 region where the mossy fiber terminal fields exist will be imaged to monitor $[Ca^{2+}]_i$ changes. In design the experiments are identical to the ones carried out by the Smith group in organotypic cultures of the hippocampus. If signals such as the ones measured by these authors are recorded, control experiments will be carried out to establish that the $[Ca^{2+}]_i$ signals result from neuronal activity. One of the most interesting possible outcomes of these experiments would be the understanding of the extent of glial cell signalling in response to neuronal activity. This knowledge will be crucial in order to reveal the functional significance of such signalling. Two other projects are planned and would follow these experiments. One involves studies of $[Ca^{2+}]_i$ changes in dendritic spines in hippocampal interneurons and the second a fluorescence recovery after photobleaching (FRAP) study to investigate the extent of coupling and its regulation in glial cell networks in the brain slice preparation.

B. Pineal cell excitability and control of melatonin secretion.

This Section has been interested in the control of cellular excitability and melatonin secretion in mammalian pineal glands by neurotransmitters. We have described a novel signal transduction mechanism for the VIP receptors in rat pineal cells and recently have identified a novel mechanism of modulation of a nonselective cation channel by cAMP in these cells in response to adrenergic and peptidergic receptor activation. The Section has the capabilities and necessary instrumentation for simultaneous recording of electrical and fluorescence signals from cells which was set up by Dr. Nissim Darvish.

A number of studies have been planned following up the work begun in the previous year. These, however will await the return of Dr. Nissim Darvish who left the Section on extended leave of absence due to the bad health of his wife. The studies will begin upon his return or in case of his decision not to return upon finding another suitable postdoctoral scientist.

1 Ion channels that regulate pineal cell excitability (Nissim Darvish)

We plan to be focused on the regulation of membrane excitability and meltonin secretion by neural control ie. activation of neurotransmitter receptors. The major receptor systems that are involved in the regulation of pineal function are the adrenergic receptors via cAMP and Ca^{2+}, and peptidergic systems including VIP and PACAP. These receptor systems also are transduced through cAMP and Ca^{2+} dependent mechanisms. Circadian control of results in melatonin secretion over prolonged periods of time, typically, from approximately 4:00 PM to 3:00AM each night. The cellular mechanisms involved in this long lasting excitability without desensitization will be the focus of our studies.

(I) The cAMP modulated nunselective cation channel

The novel mechanism of modulation of the nonselective cation channel we discovered can provide such a long lasting increased Ca^{2+} influx under resting membrane potentials and sustain excitability. In preliminary experiments, Dr. Darvish described oscillatory changes in membrane conductance over an hour with norepinephrine. The molecular mechanism of this change is not understood and we plan to characterize this phenomenon. In addition, we plan to characterize the cGMP gated cation channel and the two separate K^+ conductances identified in these cells by Dr. Darvish.

(ii) The cGMP gated cation channel.

Patch clamp techniques in conjunction with $[Ca^{2+}]_i$ measurements have identified a cGMP gated cation channel in rat pineal cells. Perforated patch experiments will be carried out to establish that VIP can activate this channel in these cells. Under voltage clamp conditions, VIP evoked membrane currents will be measured to rule out the possibility that VIP responses are mediated by membrane depolarization. Dose response relationship curves will be constructed for these VIP induced currents under voltage clamp conditions. Secondly, the depolarization induced by VIP-will be measured in current clamp. In order to investigate if the VIP currents are due to cyclic GMP generation, currents induced by 8-bromo-cyclic GMP will be measured in perforated patch experiments. The pharmacological characteristics of the currents induced by both VIP and cyclic GMP will be studied and compared. l-cis-diltiazem will be used as the antagonist to verify our earlier identification of the channel. Macroscopic current recordings will establish kinetics of channel gating and inactivation if any. Using the perforated patch technique with the input resistance of the cell being high (>1.0 GΩ), and since the pineal cells are small, single channel currents will be recorded under steady state conditions. Channels will be activated using cyclic GMP or VIP. From this data, we will analyze the dose dependent activation of the single channel and the Hill coefficient of activation. Furthermore, kinetic parameters such as mean open time, mean close time and open probability will be measured. Channel mean open time will be measured at various times after addition of cyclic GMP to establish the kinetics of inactivation of the channel. Using this paradigm, we will investigate the mechanism of inactivation. Ion selectivity for channel permeability will be established using I/V plots constructed in ion replacement experiments and calculating permeability ratios. Sensitivity to

l-cis-diltiazem of the channel will be determined.

(iii) Outward K^+ channel

Whole cell patch experiments and single channel conductance measurements will be utilized to investigate the biophysical characteristics of this channel. Initially the single channel conductance and ion selectivity will be established from I/V plots derived from experiments under different ionic conditions. Voltage dependence of the channel will then be investigated using conventional electrophysiological methods. From this data channel gating kinetics will be derived. Since this channel is outwardly rectifying as shown in our preliminary experiments at the single channel level, we intend to explore the mechanism of rectification (ie. Ca^{2+}, Na^+, H^+ etc.). Since this channel is not blocked by conventional K+ channel blockers other than cobalt we would explore other candidate blockers. Finally, based on our preliminary results potential modulation of the channel by Ca^{2+}, cyclic AMP and cyclic GMP will be investigated. Cyclic nucleotide dependent effect will be investigated to differentiate direct effects from effects mediated via phosphorylation of the channel protein through protein kinase activation.

(iv) Inward K^+ channel

Biophysical characterization of the inward K+ channel will be carried out in detail in whole cell and isolated patch experiments. These include single channel conductance and ion selectivity from I/V relationships measured under different ionic conditions. Channel open probability and its relationship to membrane potential will be investigated as well as the dependence on K+ concentration out side. Pharmacological studies will characterize the blocking action by Ba^{2+} ions. The mechanism of inward rectification of the channel will be studied and candidate agents such as Mg^{2+} and polyamines will be tested. Finally channel modulation by putative modulators such as ATP and Ca^{2+} will be examined.

Secretion of melatonin by pineal cells is tightly regulated by adrenergic input from the superior cervical ganglion. Activation of adrenergic receptors is coupled to increase in cellular Ca^{2+}, cyclic AMP, and cyclic GMP. The cellular physiology of coupling these second messengers to melatonin production and secretion is currently unknown. It is likely that the two K+ channels described here play a crucial role in not only controlling cellular excitability but also in regulating the circadian control of melatonin production and secretion. Experiments to study modulation by adrenergic agents and their second messengers are expected to reveal underlying mechanisms that control pineal function.

Publications :

Yagodin S, Holtzclaw L, Russell JT. : Subcellular calcium oscillators and calcium influx support agonist- induced caqlcium waves in cultured astrocytes. Mol.Cell. Biochem. 1995; 149-150 : 137 - 144

Holtzclaw, L. A., Gallo, V. and Russell, J. T., AMPA receptors shape Ca2+ responses in cortical oligodendrocyte progenitors and CG-4 cells. J. Neurosci. Res. 1995; 42: 124 - 130.

Gallo, V. and Russell, J. T., Excitatory aminoacid receptors in glia different subtypes for distinct functions? J. Neurosci. Res. 1995; 42: 1 - 8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02000 05 LCMN

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Neurotransmitter Receptors in Glia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Vittorio Gallo	Visiting Scientist	LCMN, NICHD	12 months
Others:	M. Pende	Visiting Fellow	LCMN, NICHD	12 months
	L.J. Chew	Visiting Fellow	LCMN, NICHD	12 months
	S. Scherer	IRTA Fellow	LCMN, NICHD	12 months
	F. Huang	IRTA Fellow	LCMN, NICHD	12 months
	C. Ghiani	Special Volunteer	LCMN, NICHD	6 months

COOPERATING UNITS *(if any)*

Dr. R. Armstrong, Department Anatomy & Cell Biology, USUHS, Bethesda, MD.

LAB/BRANCH

Laboratory of Cellular and Molecular Neurophysiology

SECTION

Unit on Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.5	5.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The molecular and functional properties of neurotransmitter ligand-gated ion channels in glial cells were studied, in order to understand their regulation and function in the brain. Studies were carried out to: A. identify the growth factors that regulate glutamate receptor expression in glia, B. characterize the physiological role of glutamate-gated ion channels in glial development, and C. clone a glutamate receptor gene and determine the role of 5' upstream and intragenic DNA elements in the transcriptional regulation of this gene. A. Effects of growth factors on glutamate receptor gene expression. Primary cultures of rat oligodendrocyte progenitors (O-2A) were used as a model system. Kainate-and AMPA-preferring glutamate receptors are co-expressed in O-2A cells. Platelet-derived and basic fibroblast growth factors selectively up-regulated GluR1 mRNA and protein, through an increase in the gene transcription rate. This corresponded to an increase in AMPA receptor density and an increased number of glutamate-gated channels that did not contain the subunit GluR2. B. Physiological properties and functional role. AMPA receptor activation inhibits O-2A cell proliferation and prevents lineage progression at early developmental stages. These effects are due to a Na^{+}-dependent block of voltage-dependent K^{+} channels. AMPA and muscarinic receptors trigger intracellular Ca^{2+} transients in O-2A cells and stimulate Ca^{2+} -dependent phosphorylation of the transcription factor CREB. Growth factors promote CREB phosphorylation independently of extracellular Ca^{2+}. The MAP Kinase/RSK was identified as the intracellular pathway of CREB phosphorylation in O-2A cells. C. Glutamate receptor genes and analysis of their 5' regulatory region. Genes encoding kainate receptor subunits are highly expressed in cells of the oligodendrocyte lineage. The cloning of the entire rat *GRIK5* gene, which encodes the kainate preferring subunit KA2 was completed, including 4 kb of its upstream region. The intron-exon organization of this gene was determined, and a negative regulatory DNA element residing within intron 1 was partially characterized. The transcriptional potential of 2kb of *GRIK5* upstream region was analyzed in transgenic mice and found to be sufficient to confer tissue-specific reporter gene expression.

Project Description:
Objective:

To study the molecular and functional properties of glial neurotransmitter-gated ion channels. To understand the physiological role of glutamate-gated ion channels in glia. To study the transcriptional regulation of glutamate receptor genes in neural cell development.

Major Findings

1. Growth factor-induced regulation of glutamate receptors in oligodendrocyte progenitor cells.

Platelet-derived and basic fibroblast growth factors (PDGF and bFGF, respectively) regulate oligodendrocyte progenitor (O-2A) proliferation, migration and differentiation *in vitro* and *in vivo*. O-2A cells co-express both AMPA- and kainate-preferring glutamate receptor (GluR) subunits. Experiments in progress in collaboration with William Hayes (Department of Biology, Catholic University) have demonstrated that O-2A progenitor cells express AMPA receptor genes *in vivo*, as detected by double *in situ* hybridization. O-2A cells were studied in developing rat cerebellar cortex sections, and identified on the basis of PDGF-a receptor mRNA expression, a marker for oligodendrocyte progenitors *in vivo*. PDGF-a receptor-expressing cells were found to express also GluR1-4 mRNAs.

Li Jin Chew analyzed the effects of PDGF and bFGF on the expression of GluR subunit mRNAs in cultured rat cortical O-2A cells after 3 days of treatment with the growth factors. In the absence of PDGF and bFGF, GluR1 was the AMPA subunit mRNA expressed at the lowest relative level. bFGF caused a 2-3 fold increase in steady-state mRNA levels for GluR1, 2, 3 and 4. PDGF did not modify by itself the mRNA levels for any of the AMPA subunits, but selectively potentiated the effects of bFGF on GluR1 mRNAs (5-fold increase). The kainate-preferring subunits GluR7, KA1 and KA2 mRNAs were increased (2-4 fold) by bFGF, but these effects were not modified by co-treatment with PDGF. Nuclear run-on assays demonstrated that PDGF+bFGF selectively increased the rate of GluR1 gene transcription (2.5 fold over control) within 20 hr after treatment of O-2A cells. RNAse protection assays showed that the two growth factors stimulated a selective 3-fold increase in GluR1 mRNA after 24 hr. Western-blot analysis showed that GluR1 protein levels were selectively increased (6-fold over control) by PDGF+bFGF. GluR2, 3 and GluR4 proteins were unaffected. In collaboration with Mark Fleck and Mark Mayer, functional expression of AMPA receptors was assessed by electrophysiological studies. The whole-cell recording configuration was used, in combination with rapid application of the agonist AMPA to cultured cells. Cyclothiazide was used in combination to AMPA, in order to suppress receptor desensitization and accurately measure the amplitudes of agonist-generated currents. From the amplitude of agonist-generated currents, normalized for the cell capacitance, it was concluded that AMPA receptor current densities (pA/pF) were increased nearly 5-fold in cells treated with PDGF+bFGF, as compared to untreated cells. Further, AMPA receptor channels in cells treated with PDGF+bFGF were more sensitive to voltage-dependent block by intracellular polyamines, as expected from the robust and selective enhancement of GluR1 expression. This indicated that treatment with PDGF and bFGF increased the density of AMPA receptors lacking GluR2. Our combined molecular and electrophysiological findings indicate that AMPA receptor function can be regulated by growth factor-induced changes in the rate of gene transcription.

2. Physiological role of glutamate receptors in oligodendrocyte development.

In culture, O-2A cells differentiate to oligodendrocyte with a time schedule similar to that *in vivo*. We hypothesized that the neurotransmitter glutamate may regulate oligodendrocyte development through the activation of membrane receptors expressed early on during O-2A proliferation and migration. We used *in vitro* bioassays and soluble factors, which stimulate mitosis, motility, and maturation of O-2A cells, to reproduce the events that occur during CNS development. Previous studies demonstrated that AMPA receptor activation inhibits O-2A cell proliferation and prevents lineage progression to the pre-oligodendrocyte stage. We began to study the mechanism of inhibition of O-2A cell proliferation by glutamate receptor agonists (kainate and AMPA). O-2A cell membrane depolarization by the alkaloid veratridine or by high (25-45 mM) extracellular K^+ for 24 hr inhibited O-2A cell proliferation stimulated by PDGF, bFGF or PDGF+bFGF. The two depolarizing agents also prevented O-2A cell lineage progression, as measured by counting the % of $O4^+$ cells (pre-oligodendrocytes) under different culture conditions. In O-2A cells cultured for 48 hr with growth factors, both veratridine and 45 mM K^+ (similarly to kainate and AMPA) significantly decreased the % of O-2A cells that became $O4^+$. Cristina Ghiani has began to study whether the anti-proliferative effects of GluR agonists and depolarizing agents are due to a reversible arrest of O-2A cells in G1 phase of the cell cycle. In synchronized O-2A cells, kainate treatment at G1 strongly decreased the % of cells that entered S-phase, whereas treatment during S- or M-phase itself did not affect O-2A cell proliferation.

In collaboration with Peter Knutson and Chris McBain, we determined that the voltage K^+ channel phenotype of O-2A cell changed during their development *in vitro*. In particular, delayed rectifier K^+ channels are highly expressed at the O-2A stage, but down-regulated in pre-oligodendrocytes. Conversely, inwardly rectifying K^+ currents were very small in O-2A cells, but up-regulated in pre-oligodendrocytes. This change in K^+ channel phenotype suggests that these channels may be involved in O-2A development. It was found that activation of GluRs in O-2A cells causes a reversible block of voltage-dependent delayed rectifier K^+ channels. These channels were also blocked by the selective K^+ channel blocker tetraethylammonium (TEA). Concentrations of TEA that caused a reversible block of these K^+ channels also strongly inhibited growth factor-stimulated O-2A cell proliferation. Additionally, the % of $O4^+$ cells was significantly decreased by TEA. These results indicate that inhibition of O-2A cell proliferation occurs through a glutamate receptor-mediated block of the delayed rectifier K^+ channels. This block is primarily due the increase of intracellular Na^+ ions caused by the opening of glutamate-gated channels, as shown by experiments in which extracellular Na^+ ions were substituted with N-methyl-D-glucose. It appears that Ca^{2+} ions are not directly involved in the block of K^+ channels and in the anti-proliferative effects described above, because: i) kainate- and TEA-induced K^+ channel block is Ca^{2+}-independent, and ii) the Ca^{2+} ionophore A23187 did not modify O-2A cell proliferation. Consistently with their anti-proliferative effects, both veratridine and 45 mM K^+ caused a reversible block of K channels in O-2A cells.

3. Glutamate receptor-induced Ca2+ transients and CREB phosphorylation in oligodendrocyte progenitor cells.

GluR activation causes phosphorylation of the transcription factor CREB in O-2A cells. Mario Pende demonstrated that stimulation of G-protein coupled (muscarinic) and tyrosine kinase (PDGF and bFGF) receptors also triggered CREB phosphorylation. The mechanism of CREB phosphorylation, in particular its Ca^{2+}-dependence and the intracellular signal transduction pathway, were further investigated. Anti-CREB antibodies selective for the 43 kDa Ser-133 phosphorylated form of CREB were used. CREB phosphorylation was stimulated by kainate, carbachol and bFGF. Increase of cAMP levels by forskolin caused only a weak induction of CREB phosphorylation. CREB phosphorylation by kainate and carbachol was rapid and transient (peak at 5 min), whereas the effects of PDGF or bFGF had a slower onset and remained constant for at least 30 min. In collaboration with Peter Simpson and James Russell, we studied the effects of kainate, carbachol and bFGF on intracellular Ca^{2+} transients. Kainate and carbachol stimulated transmembrane influx of the cation. Carbachol also triggered Ca^{2+} release from intracellular stores. bFGF responses were only displayed by 20% of the cells and their amplitude was several fold lower than the kainate or carbachol responses. The absence of extracellular Ca^{2+} abolished the kainate and bFGF responses, and eliminated the plateau component of the carbachol response. The intracellular Ca^{2+} chelator BAPTA abolished all responses. Omission of Ca^{2+} ions from the extracellular medium abolished kainate-induced CREB phosphorylation, strongly attenuated the effects of carbachol, and did not influence the effect of bFGF. Chelation of intracellular Ca^{2+} by BAPTA reduced the responses of all these stimuli. In conclusion, CREB phosphorylation stimulated by kainate and carbachol is dependent on transmembrane influx of Ca^{2+}, whereas the effects of bFGF depend on intracellular Ca^{2+}. Ca2+ ions directly influence the activity of many key regulatory enzymes, including protein kinases. Biochemical analysis of different protein kinase pathways (Ca^{2+}-calmodulin-dependent kinase, CaMK; protein kinase C, PKC; pp90 ribosomal S6 kinase, RSK; mitogen-activated kinase, MAPK), combined with the use of selective inhibitors demonstrated that CaMK and PKC are exclusively stimulated by Ca^{2+} influx, S6K exclusively by growth factors and RSK is stimulated by both types of signals. Consistently with previous findings that RSK is a downstream effector of the MAPK pathway, we determined that this pathway was responsible for phosphorylation of CREB triggered by kainate, carbachol and growth factors. In conclusion, the MAPK pathway can propagate membrane receptor signals to the nucleus in O-2A cells by inducing CREB phosphorylation.

4. Transcriptional analysis of the glutamate receptor gene *GRIK5* 5' flanking region

The gene *GRIK5* (glutamate receptor ionotropic kainate 5) encodes the kainate-preferring glutamate receptor subunit KA2. We have previously obtained a rat *GRIK5* clone comprising 2 kb of 5' flanking region. Steve Scherer continued the transcriptional analysis of *GRIK5* upstream region in order to characterize the DNA elements that regulate tissue-specific expression of this gene in the brain. Primer extension and RNase protection analyses demonstrated that this putative promoter elements contains a major transcription start site (nucleotide +1) approximately 225 bp upstream of intron 1, yielding a 5' untranslated region of 275 bases. A number of other transcription start sites were identified (from 110 bp downstream to 400 bp upstream of the major start site), as is typical of promoters lacking canonical CCAAT and TATAA boxes. The bacterial reporter gene chloramphenicol acetyl transferase (CAT) was placed under the transcriptional control of the 2 kb upstream region. As a stringent test of promoter activity, this construct was used to generate four independent lines of transgenic mice. All the lines exhibited CAT expression only in the brain. We used 5' deletion analysis in transiently transfected neural cells (glial cell line CG-4) to demonstrate that basal promoter and tissue specificity elements are present between nucleotides -232 and +94, and that further positive regulatory sequences are present between -989 and -375. Our findings show that 2 kb of *GRIK5* 5'-flanking sequence is both necessary and sufficient to direct its tissue-specific expression to neural cells both *in vitro* and *in vivo*.

5. Exon-intron organization of the rat glutamate receptor gene *GRIK5* and analysis of an intronic negative regulatory region.

Fei Huang isolated and analyzed the structure of the gene *GRIK5*. Six overlapping DNA fragments containing the entire *GRIK5* gene were identified in a rat genomic library. *GRIK5* is a unique gene distributed over 54 kilobases of genomic sequence. *GRIK5* is composed of 19 translated exons and one untranslated exon, ranging in size from 54 to 944 bp. Exon 20 (944 bp) of *GRIK5* contains 423 bp of coding and 521 bp of 3' untranslated region (a TGA translational stop codon is present 424 nucleotides downstream of the intron-exon boundary). The total length of *GRIK5* coding region (3.78 kb) deduced form the genomic sequence is consistent with the 3.9 kb mRNA identified by Northern blot analysis of tissue and cultured neural cell mRNAs. Based on the previously cloned rat KA2 cDNA sequence, polyadenylation of *GRIK5* mRNA occurs 23 nucleotides downstream of a canonical signal sequence (AATAAA) in exon 20 (nucleotides 917-922 from the 5' end of exon 20). Analysis of *GRIK5* genomic sequence and comparison with the cloned rat and mouse KA2 cDNAs suggest that, differently from other kainate and AMPA receptor subunit genes, neither alternative splicing nor RNA editing of this gene occur.

Previous analysis demonstrated that 2 kb of *GRIK5* 5'-flanking sequence confers tissue-specific expression on a chloramphenicol acetyltransferase (CAT) reporter gene *in vitro* and *in vivo*. Fei Huang found that the first intron of *GRIK5* (3.4 kb) interrupts the 5' untranslated region. By using transient transfection assays in CG-4 and PC12 cells, it was demonstrated that the first intron of *GRIK5* inhibited transcription of the CAT gene driven by the 2 kb 5'-flanking region of *GRIK5*. Deletion construct analysis indicated that the negative regulatory element resides within 500 bp of the 3'-end of intron 1. The effect of the negative regulatory element on *GRIK5* transcription was orientation-, position- and distance-independent, therefore, displays the features of a silencer. This element also inhibited CAT transcription driven by the heterologous b-actin and SV40 promoters in HeLa and 3T3 cells. Finally, gel shift assays demonstrated that the 500 bp fragment of *GRIK5* intron 1 selectively bound nuclear proteins isolated from neural and non-neural cells. We conclude that regulation of transcription of the kainate receptor *GRIK5* gene results from an interplay between positive elements located in the promoter region and an element that displays features of a silencer residing within the first intron.

Proposed Course of the Project:

1. The analysis of GluR subunit expression in cells of the oligodendrocyte lineage *in vivo* will be continued. We will extend our studies to all the AMPA and kainate receptor subunit genes, both by double *in situ* hybridization (PDGF-a receptor + GluR receptor probes), and by using individual probes in alternate tissue sections. In parallel experiments, we will also use anti GluR subunit antibodies in immunohistochemical experiments, to determine the GluR protein expression pattern in cells of the oligodendrocyte lineage *in vivo*. We plan to continue the analysis of the role of glutamate receptors in oligodendrocyte development by combining cell biological, as well as electrophysiological studies. Experiments in cultured cells will be aimed to: i) establish whether GluR activation and cell membrane depolarization cause G1 arrest in the O-2A cell cycle; ii) study cyclin expression during O-2A development *in vitro*, as well as cyclin regulation by GluR agonists and depolarizing agents; iii) determine the role played by Ca^{2+} ions in the anti-proliferative effects of GluR agonists and depolarizing agents; and iv) analyze the possibly different roles

played by distinct K^+ channels in O-2A cell development, by using selective channel blockers.

2. The molecular analysis of the *GRIK5* gene promoter will be continued in order to: i) define the minimal promoter elements required for tissue-specific expression; ii) identify the DNA elements directly involved in the binding of *trans*-acting factors. Further deletion analysis of the *GRIK5* promoter will be performed by transient transfection assays in CG-4 cells. Some of the deletion constructs will also be analyzed in transgenic mice. *In vitro* techniques, such as gel retardation and DNAse I footprinting assays will be performed with nuclear extracts prepared from different neural cell types expressing *GRIK5* (CG-4 cells, cerebellar granule cells, PC12 cells, oligodendrocytes, astrocytes), and from CNS tissue at different developmental stages. To determine the sequence specificity of the footprints, the effects of mutations on nuclear protein binding sites will also be examined *in vitro* as well as in transient transfection assays.

3. *GRIK5* expression will be analyzed in two different neuronal cell systems, the cerebellar granule cells and PC12 cells, with the goal of identifying growth factors or culture conditions that may regulate *GRIK5* expression during neuronal development. *GRIK5* promoter constructs will be made in a luciferase vector, and transcriptional activity will be assayed after transient transfection into cell lines or primary cell cultures. The effects of Nerve Growth Factor on *GRIK5* expression will be assayed in PC12 cells, and the effects of serum and chronic membrane depolarization (high extracellular K^+) will be analyzed in cerebellar granule cells. Finally, the DNA elements involved in this regulation will be identified by deletion analysis combined to gel retardation and DNAse I footprinting assays.

4. We will continue to analyze the role of intron 1 in the regulation of *GRIK5* gene expression, in particular we want to determine whether the negative regulatory element of this intron is involved in down-regulating *GRIK5* gene expression after birth, or in selecting its expression in distinct cell types. We will analyze *GRIK5* transgene expression in different cell types of the brain by using LacZ constructs in which the *GRIK5* promoter is linked to intron 1. Transgene expression will be studied at different embryonic and post-natal stages. In parallel experiments, we will also perform DNAse I footprinting analysis on the negative regulatory region of intron 1 with nuclear extracts from different neural cell types, and from brain tissue at different developmental stages. This set of experiments will pinpoint the DNA elements of intron 1 involved in down-regulating *GRIK5* gene expression.

Significance to Biomedical Research and Program of the Institute:

It is well established that glial cells perform a pivotal role in brain function and express a large variety of voltage-dependent and ligand-gated channels. This finding greatly increases the complexity of the cellular elements that can respond to classical neurotransmitters, because the ratio between glial cells and neurons in the mammalian central nervous system is approximately 10:1. Glutamate receptors are abundantly expressed in different glial cell types and they can mediate drastic changes in glial membrane potential. The physiological role of glutamate-gated channels in glia is not understood, but the close anatomical relationship between neurons and glia both in the developing and in the mature mammalian central nervous system indicates that also glial cells are very likely to be directly affected by glutamate in the intact brain. The appearance of glutamate receptors during early glial development and the regulation of glial proliferation and differentiation by glutamate indicate that, in addition to his role as mediator of information transfer at synaptic junctions, glutamate plays a role in shaping the development of the central nervous system. This has very important consequences for the physiology of the mature brain, in which glial cells perform crucial functions, such as myelination and metabolic support of neurons. Glutamate receptors in glial cells may also play an important role in the pathophysiology of stroke and cerebral anoxia, as already established for neurons. In addition, periventricular white matter injury, the principal variety of brain injury of the human premature infant, cause cell death of differentiating oligodendroglia and appears to involve glutamate receptors.

Publications:

Gallo V, Russell JT. Excitatory amino acid receptors in glia: different subtypes for distinct functions? J Neurosci Res 1995; 42: 1-8.

Holtzclaw L, Gallo V, Russell JT. AMPA receptors shape Ca^{2+} responses in cortical oligodendrocyte progenitors and CG-4 cells. J Neurosci Res 1995; 42: 124-130.

Levi G, Gallo V. Release of neuroactive amino acids from glia. In: Kettenmann H, Ransom BR, eds. Neuroglia. New York: Oxford University Press, 1995; 815-826.

Gallo V, Zhou JM, McBain CJ, Wright P, Knutson PL, Armstrong RC. Oligodendrocyte progenitor cell proliferation and lineage progression are regulated by glutamate receptor-mediated K^+ channel block. J Neurosci 1996; 16: 2659-2670.

Steinhauser C, Gallo V. News on glutamate receptors in glial cells. Trends Neurosci. 1996; 19: 339-345.

LDMI

NICHD Annual Report
October 1, 1995 to September 30, 1996

Laboratory of Developmental and Molecular Immunity

The LDMI studies the pathogenesis of and immunity to bacterial diseases, especially those of infants and children, in order to develop vaccines for their prevention. Since almost all pathogens of healthy individuals are pathogenic for humans only, the LDMI directs most of its efforts at bringing their investigational vaccines to clinical evaluation for safety, immunogenicity and, ultimately, efficacy, as soon as possible. The spirit that guides most of the research efforts of the LDMI is "the proper study of mankind is man."

Enteric Bacterial Infections: Vaccine development for enteric pathogens has been limited because of the lack of valid animal models and controversy about the host immune mechanism(s). We proposed that a critic (protective) level of serum IgG to surface polysaccharides alone is sufficient to confer immunity to these pathogens. The pivotal argument to support this hypothesis is that serum IgG exudes onto epithelial surfaces, including those of the intestine, and lyses the inoculum of the pathogen. The development of conjugates for enteric pathogens was stimulated by the observation that these types of vaccines were highly effective in inducing protective levels of both serum IgG polysaccharide and carrier protein antibodies in infants and young children.

The Vi capsular polysaccharide of *Salmonella typhi* (poly $\alpha(1\rightarrow4)$-D-Gal*p*NAcA, *N*-acetylated at C2 and *O*-acetylated at O-3). Vi has now been licensed by the FDA in about 40 countries and the World Health Organization has published Requirements for this new typhoid vaccine. The limitations of Vi as a vaccine, similar to other capsular polysaccharides, are its age-dependent and T-cell independent immunologic properties. We developed methods to covalently bind Vi with carrier proteins using heterobifunctional thiolating linkers. The carboxyls of Vi were derivatized with cystamine (~1-3%) and this derivative was linked to proteins with the heterobifunctional reagent *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). The proteins chosen for the clinical trial were the B-subunit of heat-labile toxin (LT-B) from *Escherichia coli* and the recombinant exoprotein A from *Pseudomonas aeruginosa* (*r*EPA). When injected subcutaneously in saline solutions, the Vi conjugates elicited higher antibody levels than Vi alone and a booster response in guinea pigs and mice. Volunteers, 18-40 years old, were injected twice, 4-6 weeks apart, with the Vi conjugates or Vi. None of the 51 vaccinees had fever or significant local reactions. The Vi conjugates elicited higher and longer lasting antibody levels than Vi alone ($p=0.01$). Vi-*r*EPA elicited slightly higher levels of Vi antibodies than did Vi-LT-B at all intervals after injection, but these differences were not statistically significant. Similar to other polysaccharide-protein conjugates in adults, reinjection did not elicits a booster effect. Phase 2 clinical trials of Vi-*r*EPA have been completed and were shown to be safe (not significant reactions and immunogenic in 2 to 5-year-old children in Vietnam. Clinical efficacy trials are underway. Pectin, a plant polysaccharide, is mostly a homopolymer of (1→4)-*α*-*D*-Gal*p*A with ~5% neutral sugars and has an average molecular weight ~400 kDa. It differs from Vi in that it lacks an *N*-acetyl at the C2 and *O*-acetyl at the O-3 positions. There is no serological cross-reaction between pectin and Vi. Pectin, when acetylated at O-2 and O-3, is antigenically identical to Vi in double immunodiffusion. Unlike Vi, *O*-acetylated pectin (OAcPec) is not immunogenic in mice, probably because of its low molecular weight. OAcPec conjugated to tetanus toxoid following the procedure for Vi conjugates (*vide supra*) elicited Vi antibodies in mice: reinjection elicited a booster response. OAcPec has advantages over Vi because it is easily prepared, can be measured by standardized colorimetric assays and forms more soluble conjugates with proteins. The use of a plant polysaccharide, pectin, as an immunogen for prevention of a systemic infection caused by a capsulated pathogen, *S. typhi* provides a novel approach to improve the preparation and immunogenicity of polysaccharide-based vaccines.

We tried another synthetic scheme for Vi and di-O-acetyl pectin conjugates using carbodiimide-mediated synthesis with adipic acid dihydrazide (ADH) as the linker. First, hydrazide groups were introduced into BSA or *r*EPA by treatment with ADH and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. The resultant adipic acid hydrazide derivatives (AH-proteins) were bound to Vi and OAcP by treatment with EDC. The immunogenicities of Vi or OAcP alone or as a conjugate were evaluated in young outbred mice and guinea pigs by subcutaneous injection of 2.5 μg or 5.0 μg of polysaccharide respectively. All conjugates were significantly more immunogenic than Vi or OAcP alone and induced booster responses with 5-25-fold increases of antibodies. Vi conjugates were significantly more immunogenic than their OAcP analogues. Vi and OAcP conjugates synthesized by this scheme will be evaluated clinically.

The surface polysaccharides of enteric pathogens, such as the capsular polysaccharide of *S. typhi* (Vi) or the O-specific polysaccharide domain of lipopolysaccharides of nontyphoidal *Salmonella*, *Shigella*, or *E. coli*, are both essential virulence factors and protective antigens. These surface polysaccharides "shield" the bacterium from the lytic actions of serum complement.

S. paratyphi A, the second most common cause of enteric fever in Southeast Asia, is a habitant of and a pathogen for humans only. Accordingly, *S. paratyphi* is being studied as a model for all nontyphoidal *Salmonella*. The O-specific polysaccharide of *S. paratyphi* A is composed of a trisaccharide. Of note is that the O-3 of the *β*-L-rhamnose is acetylated. Conjugates composed of the detoxified LPS of *S. paratyphi* A were prepared by several schemes. LPS was detoxified with acetic acid or with hydrazine: the latter removed *O*-acetyls from the O-specific polysaccharide. The detoxified LPS were activated with cyanogen bromide (CNBr) or with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) and bound to TT with or without a spacer. Solutions of 2.5 μg saccharide, alone or as a conjugate, were injected subcutaneously into young mice and LPS and TT antibodies measured by ELISA. Only conjugates with *O*-acetyls elicited serum IgG anti-LPS with bactericidal activity. There were no statistically significant differences between LPS antibody levels elicited by conjugates synthesized with or without a spacer. The conjugate with O-specific polysaccharide activated by CDAP and bound to TT without a spacer elicited the highest level of TT antibodies. Clinical evaluation of *S. paratyphi* A conjugates is planned.

The O-specific polysaccharide of *S. sonnei* was bound to *P. aeruginosa* exoprotein A (*r*EPA) and adult volunteers from the Israel Defense Force vaccinated with 25 μg of the investigational vaccine. None of the 576 volunteers experienced significant local symptoms or fever. In a blinded, randomized, vaccine controlled trial, the *S. sonnei* demonstrated efficacy (74%, 95% CI 27-100, p=0.006) against culture-positive infection 77 to 155 days after vaccination. In one company, the *S. sonnei* conjugate showed significant protection (42%, 95% CI 4-81, p=0.043) against culture-positive shigellosis occurring 1 to 17 days after vaccination. This unexpected finding indicates that this type of conjugate could be useful in controlling an epidemic since many of the infections in that company were present before or soon after vaccination. Vaccinees who developed shigellosis caused by *S. sonnei* had significantly lower serum IgG and IgA antibody response to the homologous LPS compared to those who were vaccinated and did not contract disease. *Shigella* and *E. coli* may be considered as one species and these data provide evidence that our conjugate vaccines, designed to induce serum IgG anti-LPS, will be effective against enteric bacterial infections.

Immunoprophylaxis of *E. coli* O-157 is sought because this organism is a cause of severe enteritis and the extraintestinal complication of hemolytic uremic syndrome especially in children. Although *S. dysenteriae* type 1 and *E. coli* O-157 have a pathogenicity and extraintestinal complications in humans, *E. coli* O157:H7, and not *S. dysenteriae* type 1, live in cattle without causing symptoms. Accordingly, there is little information about the host mechanism/s of immunity and vaccine development because there are no valid animal models for infections caused by *E. coli* O-157 or *S. dysenteriae* type 1. *E. coli* and *Shigella* should be considered as one Genus. There is indirect evidence from the study of *Shigella* to suggest that a "critical" level of serum IgG antibodies to the O-specific polysaccharide of *E. coli* O-157 may confer immunity to enteritis caused by this pathogen, *viz*: 1) immunity following shigellosis is LPS-specific; 2) the level of serum IgG O-specific polysaccharide antibodies predict resistance to shigellosis; and 3) preliminary data show that vaccination with a polysaccharide-protein conjugate prevents infection with *S. sonnei* (*vide infra*). To-date, we prepared O-specific polysaccharide conjugates for *E. coli* O-157 and for the second most common cause of HUS in children *E. coli* O-111. Both experimental vaccines had clinically acceptable levels of "endotoxin" and, in mice, both conjugates induced IgG with bactericidal activity for their homologous organisms.

E. coli O-111, of varying H types and virulence factors, cause enteritis throughout the world especially in young children: this pathogen is the second most common cause of HUS in the U.S. Serum antibodies to the O-specific polysaccharide of O-111 LPS protect mice and dogs against infection. The O-111 O-specific polysaccharide is composed of a pentasaccharide repeat unit with two colitoses bound to the O-3 and O-6 of glucose in a trisaccharide backbone identical to that of *S. adelaide* (O35), another enteric pathogen. O-111 O-specific polysaccharide was prepared by treatment of its LPS with acetic acid (O-SP) or the organic base hydrazine (DeA-LPS). The O-SP had reduced concentration of colitose. These products were derivatized with adipic acid dihydrazide (ADH) or thiolated with *N*-succinimidyl-3(2-pyridyldithio) propionate (SPDP). The four derivatives were covalently bound to tetanus toxoid (TT) by carbodiimide-mediated condensation or with SPDP to form conjugates. Immunization of mice by a clinically acceptable route showed that hydrazine-treated O-specific polysaccharide conjugate bound to tetanus toxoid by adipic acid dihydrazide elicited the highest level of LPS antibodies. This method spared the colitose residues from acid-catalyzed hydrolysis.

Phase 1 clinical studies with O-157 conjugates showed that they were safe (no fever or significant local reactions) and they induced significant rises of IgG antibodies with bactericidal activity in all 75 adult volunteers. Adults require only one injection to achieve maximal levels of vaccine-induced antibodies. Clinical studies in children are planned as part of our objective to include these *E. coli* O-157 and O-111 vaccines in the routine vaccine formulation.

Synthetic Approach: There are many reasons, based upon immunologic studies, that synthetic saccharides of defined length and homogeneous sites for conjugation would improve the immunogenicity of our conjugate vaccines. A number of oligosaccharides corresponding to the O-specific polysaccharide of S. dysenteriae type 1 have been synthesized and their binding properties to monoclonal antibodies and their conformation, compared to the LPS extracted from the bacteria, were examined by several physico/chemical methods. In addition, synthetic antigens for this pathogen containing dimers, trimers and tetramers of the tetrasaccharide repeating unit corresponding to octa-, dodeca- and hexadecasaccharides, have been synthesized. A new heterobifunctional linker containing a free carboxyl group and a masked aldehydo moiety was developed and used for the covalent attachment of the synthetic saccharidesz to human serum albumin at multiple sites on the protein. Briefly, the amino group installed at the terminus of the aglycon of the synthetic saccharides was acylated with the spacer moiety using carbodiimide activation. Next the protecting groups of the aldehydo function of the linker were removed by mild acid hydrolysis. Finally, the saccharide-spacer construct was anchored to the protein by reductive amination. In a collaborative study the monosaccharide components of the O-specific polysaccharide of S. sonneiI have been prepared and the synthetic approaches to the disaccharide repeating unit and their multiplets are underway.

A method was developed for the rapid isolation of pure B subunit of Shiga toxin using affinity chromatography based on the blood group P1 antigenic trisaccharide. This trisaccharide, which is a receptor-binding analog of Shiga-toxins, was chemically synthesized and was covalently attached to an agarose-based solid support. The B subunit of *Shigella* was prepared from the supernatant of a culture of the recombinant nonvirulent *V. cholerae* strain 0395-N1 (prepared by Drs. Arthur Donahue-Rolfe and Stephen Calderwood). This B subunit was used as a carrier protein for *E. coli* O157 O-specific polysaccharide and this conjugate elicited polysaccharide and neutralizing antibodies to Shigella toxin. Clinical studies with this new conjugate are planned.

Tuberculosis: Animal and human serum induced by vaccination with pneumococcus type 12F (Pn12F) organisms in a horse and rabbits or in humans by a Pn12F polysaccharide (PS)-diphtheria toxoid conjugate, kill *Mycobacterium tuberculosis* in the presence of complement and human blood neutrophils. The specificity of bactericidal activity was confirmed by adsorption of the Pn12F antisera with either Pn12F PS, dextran 1299, or kojipentaose. Rabbit anti-pneumococcal type 6A (Pn6A), anti-lipoarabinomannan (LAM) or human pre-vaccination sera did not exert this opsonophagocytic killing. Purified human Pn12F PS antibodies elicited this specific bactericidal effect and were seen to bind to the surface of *M. tuberculosis* organisms by immuno-electron microscopy. These findings indicate that *M. tuberculosis* has a surface polysaccharide that is vulnerable to serum antibody-initiated complement-dependent opsonophagocytic killing. Since this bactericidal activity can be a correlate for immunity, we plan to evaluate antibodies to the surface polysaccharides of M. tuberculosis for their ability to prevent primary tuberculosis pneumonia.

Pertussis: In addition to the finding that the NICHD monovalent pertussis toxoid is highly safe and confers immunity to pertussis, the collaborative program with Drs. Birger Trollfors, John Taranger and Teresa Lagergard in Goteborg, Sweden, has unearthed two important observations. First is that there is an artifact created when serologic tests for pertussis use components in the vaccine. The criteria for diagnosis of the World Health Organization requires ≥21 days of paroxysmal coughing and isolation of *Bordetella pertussis* or a significant rise of serum IgG anti-pertussis toxin or anti-filamentous hemagglutinin. We showed that vaccination with pertussis toxoid reduces the yield of *B. pertussis* and, therefore, greater reliance is placed on the serologic diagnosis of pertussis. About 90% of pertussis patients, both vaccinated with the pertussis toxoid or controls (recipients of diphtheria and tetanus toxoids, respond with a significant rise of anti-FHA. In contrast, only about 25% of vaccinees respond with a rise of anti-pertussis toxin whereas about 90% of controls respond to this antigen. Accordingly, the serologic assays artificially lower the number of cases among recipients of multivalent vaccines of which contain pertussis toxoid and FHA. Second is the demonstration of "herd" immunity (protection of the non-vaccinated) among family members of vaccinees. This last finding, along with the demonstration that widespread vaccination of the remainder of children ≤5 years of age in Goteborg has resulted in a statistically significant decline in the number of cases in that city, provide further evidence that pertussis toxoid alone is both necessary and sufficient for the new pertussis vaccine and that this safe product is suitable for addition to diphtheria and tetanus toxoids for routine vaccination of adults in order to eliminate that reservoir of *B. pertussis* that remains in that age group.

Project Numbers - Annual Report 1996

LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY (LDMI)

ZO1 HD 01301-14	Human Immune Response to Polysaccharide-Protein Conjugate Vaccines Rachel Schneerson, M.D.
ZO1 HD 01308-13	Conjugate-Induced Polysaccharide Antibodies Shousun C. Szu, Ph.D.
Z01 HD 01311-03	A Polysaccharide Vaccine for a Mycobacterium-Tuberculosis John B. Robbins, M.D.
Z01 HD 01312-02	Isolation and Purification of Subunit B of Shiga Toxin Vince Pozsgay, Ph.D.
Z01 HD 01313-02	Synthetic Vaccine Against Shigellosis Vince Pozsgay, Ph.D.
Z01 HD 01314-02	Analysis and Synthesis of Carbohydrate Antigens of Mycobacterium Tuberculosis Vince Pozsgay, Ph.D.
Z01 HD 01315-02	Modulation of Protein and Cell Functions by Heparin/Heparan Sulfates Audrey L. Stone, Ph.D.

ZO1 HD 01316-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01301-14 LDMI

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Human Immune Response to Polysaccharide-Protein Conjugate Vaccines

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: R. Schneerson Research Medical Officer LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)*

LCDB, NIDDK (J. Shiloach); SUNY Downstate Medical Center, N.Y. (G. Schiffman); Carolinas Medical Center, Charlotte, N.C., (J.C. Parke, Jr.); National University Hospital, Iceland (J. Jonsdottir); LCI, NIAID (J.

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-2720

TOTAL STAFF YEARS: 5.8	PROFESSIONAL: 2.3	OTHER: 3.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The surface polysaccharides of bacterial pathogens, including capsular polysaccharides and lipopolysaccharides, serve as protective antigens. The immunologic properties of these bacterial polysaccharides, namely their age-related and T-cell independent immunogenicity, limit their use as vaccines. Covalently attachment to medically-useful proteins to form conjugates, both increases their immunogenicity and confers T-cell dependent properties to these polysaccharides. The capsular polysaccharides of Streptococcus pneumococcus type 6B have been bound to tetanus toxoid and evaluated clinically. S. aureus type 5-rEPA was evaluated in end stage renal disease patients; type 5 antibodies of the three major Ig classes rose significantly though to a lesser degree compared to healthy volunteers. As expected, there was no booster response to reinjection. These vaccine-induced antibodies had opsonophagocytic activities. Pn6B-TT was evaluated in patients with sickle cell disease, healthy infants at 3, 4 and 6 months of age or at 7 and 9 months of age. Type specific antibodies of the three Ig classes, with booster responses, were induced. The magnitude of these responses was lesser than of Hib-TT. All conjugates were safe, with only minor local reaction. The LPS of shigellae was detoxified, their O-specific polysaccharides bound to bacterial toxoids and their immunogennicity in mice found to be satisfactory. In Phase 1 and Phase 2 studies, these conjugates of the O-specific polysaccharides were safe and immunogenic: LPS antibody levels elicited by the investigational conjugates were similar to those in recruits convalescent from shigellosis vaccine-induced IgG persisted at high levels for two years. In preliminary though statistically significant studies, a S. sonnei-rEPA conjugate protected against shigellosis caused by this pathogen. A phase 2 study of S. sonnei and S. flexneri in children showed both conjugates to be highly safe and immunogenic. Phase 3 trials are being planned.

PHS 6040 (Rev. 5/92)

Others:	J.B. Robbins	Chief	LDMI, NICHD
	V. Pozsgay	Visiting Scientist	LDMI, NICHD
	D. Pavliakova	Visiting Associate	LDMI, NICHD
	D.A. Bryla	Statistician	LDMI, NICHD
	N.W. Tolson	Biologist	LDMI, NICHD
	O.L. Barrera	Adjunct Technician	LDMI, NICHD
	F.D. Majadly	Adjunct Technician	LDMI, NICHD

Cooperating Units: Bennett); LMB, NCI (D. Fitzgerald); WRAIR, Washington, D.C. (D.N. Taylor); IDF, Israel (D. Cohen); Sheba Medical Center, Israel (J. Passwell); Children's Hospital of Michigan, Detroit, MI (S. Sarnaik)

Project Description:

The surface polysaccharides of bacterial pathogens that cause systemic infections are both essential virulence factors and protective antigens. Primary pathogens cause systemic infections in otherwise healthy individuals. Their surface polysaccharides may be capsular polysaccharides or lipopolysaccharides. Most opportunistic pathogens also have surface polysaccharides that may be protective antigens. The immunologic properties of polysaccharides limit their usefulness as vaccines. For capsular polysaccharides there is: 1) Age-related serum antibody response. Immunization of infants elicits few responses of mostly IgM and of short duration. "Protective" levels start to be achieved in two year-olds and an adult response develops at ~6 - 12 years of age; 2) T-cell independence. Most polysaccharides do not elicit a booster response at any age. The situation for lipopolysaccharides (LPS) is slightly different. LPSs are composed of three domains: O-specific polysaccharide - Core - Lipid A. The latter is attached to the outer membrane and the pharmacologic properties of the bacteria are mediated by Lipid A (pyrogenic, inflammatory, vasomotor activity [shock] and effect upon WBCs and platelets). Accordingly, the lipid A alone or as part of LPS is not suitable in a vaccine; and 3) the O-specific polysaccharide is the region in contact with the host and is a protective antigen but by itself is not immunogenic.

Objectives:

Conjugates are more immunogenic than systemic infections: Vaccines more immunogenic than disease. When bound to foreign proteins, especially inactivated and purified vaccine components (tetanus and diphtheria toxoids), the immunologic properties of polysaccharides improve. One of our objectives is to improve the immunogenicity of polysaccharide-based conjugates. One important variable has been the molecular weight of the polysaccharide component. This has been controversial, with some investigators suggesting an "optimal" size between high molecular weight, perhaps about 40,000D, and "oligosaccharides." Our laboratory and others have shown that conjugated higher molecular weight polysaccharides elicit higher levels of antibodies. There is, however, the technical problem that high molecular weight polysaccharides tend to form more insoluble and technically unsatisfactory conjugates.

Another problem is the poor immunogenicity of the polysaccharides such as the capsular polysaccharides of pneumococcus type 6 and the poly $\alpha(2\rightarrow8)$Neu*p*NAc of Group B meningococci and E. coli K1: both of these capsules are associated with important and common pathogens. As conjugates, these polysaccharides have proven to be lesser immunogens, than other polysaccharide conjugates, though more immunogenic their unconjugated.

Pertussis toxin will certainly be a component, if not the sole component of the new acellular pertussis vaccines. Since it is anticipated that additional carrier proteins will be required for a multivalent polysaccharide conjugate formulation, pertussis toxin should be a useful candidate. Preliminary experiments showed that polyanions, including medically useful capsular polysaccharides, combine and precipitate with pertussis toxin. Pertussis toxin is not soluble at pH from about four to nine making it difficult to use for conjugation. Further, the neutral polysaccharide, pneumococcus type 14, is also from a common and important pathogen for children. Conjugates formed between pneumococcus type 14 and pertussis were synthesized and were not toxic in vitro and in mice. The type 14-pertussis toxin conjugate elicited both type 14 and pertussis neutralizing antibodies in mice. The clinical safety and efficacy of such a conjugate must now be evaluated.

A heterofunctional, fully protected tetrasaccharide was synthesized which corresponds to a complete repeating unit of the O-specific polysaccharide of S. dysenteriae type 1 and was used as a building block for the preparation of higher-order saccharides. A dimer, a trimer and tetramer of this repeating unit were assembled, corresponding to octa-, dodeca- and hexadeca-saccharide fragments of the O-specific, polysaccharide and their binding to Mabs to S. dysenteriae type 1 studied.

Lastly, the LDMI has demonstrated good immunogenicity and preliminary efficacy of Shigella sonnei O-specific polysaccharide-protein conjugates in armed forces recruits of the Israel Defense Force. The LDMI has now began evaluating these investigational vaccines in children, the age group that suffers the highest incidence, morbidity and mortality caused by shigellae and other enteric pathogens.

Methods Employed:

The capsular polysaccharides of pneumococcus type 6B and Group B streptococci were purified and characterized according to methods established in our laboratory. The LPS was extracted from Plesiomonas shigelloides (identical to S. sonnei) and S. flexneri type 2a, purified by hot phenol extraction and their O-specific polysaccharide purified by acid hydrolysis. The final products were purified by gel filtration and their purity and biosafety assayed by established methods.

Clinical evaluation of conjugate vaccines at the Clinical Center, NIH, the Walter Reed Army Institute of Research and the Israel Defense Force and the Sheba Medical Center proceeded after approval by the NIH, FDA, IRBs of the above institutions.

Serum antibodies to capsular polysaccharides, O-specific polysaccharides and to the carrier proteins were measured by a standardized ELISA.

In order to avoid the potential toxicity of pertussis toxin as a conjugate, a non-toxic protein will be used. A genetically engineered strain of B. pertussis synthesizing a cross-reacting non-toxic mutant pertussis toxin has been cultivated and initial studies of it purification and methods for improving its solubility are underway. Since the mutation has been created in the enzymatically active site, the techniques of affinity chromatography, used successfully for the native toxin are not applicable.

Major Findings:

In a preliminary study of recruits of the Israel Defense Forces, the S. sonnei-*r*EPA conjugate vaccine was shown to confer type-specific protection against diarrhea and fever. A more extensive placebo-controlled, randomized blinded study showed an efficacy of about 75%. A phase 2 study (safety and immunogenicity in children and then, if the results are satisfactory, in infants) has been approved and started by the Pediatrics Department, Sheba Medical Center, Israel. Prelminary epidemiologic analysis of enteric infections has started in a rural area of Vietnam in order to provide information about Shigella types that are important but not common in the U.S. or in countries like Israel.

S. aureus type 5-rEPA injected into 17 end-stage-renal disease patients induced type 5 antibody rises in the three major Ig classes, mostly in IgG. Maximal levels at six weeks, were not boosted by an additional injection at this time. Fold increases and GM levels were about one-half those of normal volunteers, but at six months, the GM IgG levels were 10-fold higher than the preimmunization levels.

Pn6B-TT and GBS III-TT were found to be injected at about half the intended dose due to the conjugates binding to the vials, GBS III-TT was also shown to break down over a two year period. Pn6B-TT injected into adult and chronic obstructive pulmonary disease individuals induced antibodies of isotypes and opsonic activities similar to those induced by the 23 valent polysaccharide vaccine. This Pn6B conjugate lot was injected into infants at 3, 4 and 6 months of age (n=21) or at 7 and 9 months of age (n=19). In the younger infants only two achieved the estimated protective level after the primary immunization, in the older infants ten reached this level. Booster injections at 18 months induced high antibody levels (5-15-fold the estimated protective level) in most children. This lot was also injected into 17 infants with sickle cell disease. The antibody responses of these infants was similar to those of the healthy infants.

GBS III-TT injected into child-bearing age females induced GM IgG anti-type III levels similar to the reported for the polysaccharide alone, with a decline of one-third at one year.

Proposed Course:

Methods to improve the immunogenicity of conjugate vaccines, especially of lesser immunogenic polysaccharides will be investigated. Clinical lots of Haemophilus influenzae type a and meningococcus group B will be prepared.

Significance to Biomedical Research and the Program of the Institute:

The above diseases are major causes of morbidity and mortality in the U.S. and worldwide. In particular, shigellosis is a major cause of stunted growth of children throughout the world. In addition to providing an effective vaccine for preventing these infections, the LDMI research program is attempting to confirm that vaccine-induced serum antibodies, especially of the IgG class, can be a protective mechanism against enteric infections including both bacterial and viral pathogens.

Publications:

Claesson BA, Trollfors B, Anderson PW, Johansson J, Taranger J, Schneerson R, Robbins JB. Serum antibodies in 6-year-old children vaccinated in infancy with a Haemophilus influenzae type b-tetanus toxoid conjugate vaccine. Pediatr Infect

Dis J 1996; 15:170-2.

Welch PG, Fattom A, Moore J, Jr, Schneerson R, Shiloach J, Bryla DA, Li X, Robbins JB. Safety and immunogenicity of Staphylococcus aureus type 5 capsular polysaccharide-Pseudomonas aeruginosa recombinant exoprotein A conjugate vaccine (SA5-rEPA) in patients on hemodialysis. J Am Soc Nephrol 1996; 7:247-53.

Madore DV, Anderson P, Baxter DB, Carlone GM, Edwards KM, Hamilton RG, Holder, P, Kayhty H, Phipps DC, Peeters CCA, Schneerson R, Siber GR, Ward JI, Frasch CE. Interlaboratory study evaluating quantitation of antibodies of Haemophilus influenzae type b polysaccharide by enzyme-linked immunosorbent assay. Clin Diagn Lab Immunol 1996; 3:84-8.

Robbins JB, Schneerson R, Szu SC. Polysaccharide-protein conjugate vaccines. In: Plotkin S, Fantini B, eds. Vaccina, vaccination and vaccinology: Jenner, Pasteur and their successors. Paris: Elsevier, 1996; 135-43.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01308-13 LDMI

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Conjugate-Induced Polysaccharide Antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S.C. Szu	Research Chemist	LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)* Walter Reed Army Institute of Research, Washington, D.C. (D.N. Taylor); LCDB, NIDDK (J. Shiloach); Carolinas Medical Center, Charlotte, N.C. (J.C. Parke, Jr.)

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Unit on Molecular Biophysics and Biochemistry

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.4	4.2	1.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Serum antibodies to the surface polysaccharides of Gram-negative enteric bacteria confer protective immunity against these pathogens. Vi capsular polysaccharide of Salmonella typhi was conjugated with protein to improve its immunogenicity. Phase 1 studies confirmed the superior immunity of recombinant exoprotein A of Pseudomonas aeruginosa (rEPA) bound to Vi. A phase 2 study with this investigational vaccine has been completed for Vietnam, an area with a high attack rate of typhoid fever. A semi-synthetic Vi antigen using plant polysaccharide, pectin, was prepared and showed identical antigenicity as the native Vi. A conjugate, composed of a di-O-acetylated pectin as a vaccine for typhoid fever, has been prepared for a phase 1 study in Vietnam for comparative immunogenicity with Vi conjugates. A different synthetic scheme for preparing Vi conjugates was devisednd the new product characterized for its immunogenicity in mice. Escherichia coli O157, considered as an important emerging pathogen in the U.S., causes hemolytic uremic syndrome in young children. Phase 1 study of the detoxified O-specific polysaccharide and covalently bound to rEPA elicited 100-fold antibody rise in healthy adults after four weeks and the antibodies were bactericidal. We plan a phase 2 study for therapeutic usage of hyperimmune plasma in children. Salmonella paratyphi A accounts for 10% of enteric fever in Southeast Asia. A vaccine prepare in the similar fashion as E. coli O-157 was prepared and elicited bactericidal antibodies in mice. We plan to use this conjugate as a control in our phase 2 study in Vietnam.

Others:	J.B. Robbins	Chief	LDMI, NICHD
	S. Bystricky	Visiting Scientist	LDMI, NICHD
	E.Y. Konadu	Visiting Fellow	LDMI, NICHD
	Z. Kossaczka	Visiting Fellow	LDMI, NICHD
	D.A. Bryla	Medical Statistician	LDMI, NICHD
	R.T. Funk	Chemist	LDMI, NICHD

Project Description:

Enteric bacterial pathogens are a major cause of mortality and morbidity, especially in infants and children, throughout the world. In the United States, enteric bacterial pathogens continues to cause outbreaks of diseases and admissions to hospitals. The LDMI has developed experimental data to show that the surface polysaccharides of these bacteria are both essential virulence factors and protective antigens. Most importantly, the LDMI has shown that vaccine-induced serum antibodies to these polysaccharides can exert protection and a mechanism by which this protection may occur has been proposed. Lastly, the LDMI has extended their expertise to develop polysaccharide-proteins conjugate vaccines which can easily be standardized and can be administered to infants as part of their routine schedule of immunization.

Objectives:

Enteric fevers are common and serious worldwide: the most important of which is typhoid fever. In Southeast Asia, more than 70% isolates are antibiotic resistant. The capsular polysaccharide (Vi) is both an essential virulence factor and a protective antigen of Salmonella typhi. Field trials in Nepal and in the Republic of South Africa showed that a single injection of Vi conferred about 70% protection in children ≥5 years-old and in adults. Its protective action is to elicit a critical level of serum antibodies. Vi was conjugated to proteins to increase its immunogenicity and make it suitable for routine immunization of infants against typhoid. A Phase 1 clinical trial in the U.S. showed that Vi conjugates elicited significantly higher levels of serum antibodies than the Vi alone. LDMI is planning to clinical trials of Vi conjugates in children and infants of endemic areas.

New methods to conjugate minimizing cross-linking Vi with homobijunctional linker is under development. Vi and its antigenic analogue, 2,3 O-acetylated pectin [(1→4)(α-D-Gal-pA)].

In order to understand the difference between the conformational stability of these two molecules are compared with energy minimization calculation and molecular dynamic simulation. Polysaccharides, unlike proteins, do not fold into globular conformation and usually are in extended helical forms. Replacing the *N*-acetyl group at the C2 position with the *O*-acetyl also remove the positive charge and the possible proton donor to form hydrogen bond with the neighboring carboxyl group. The distance and angles of the glycosidic bonds are calculated both in vacuum and in water to compare the solvent effect.

The relation between polysaccharide structure and its immunologic properties is important for designing an effective vaccine or in searching for cross-reactive antigens. The aforementioned pectin-Vi study serves as a good example. One other highly charged polysaccharide, the α(2→8) linked polysialic acid is the surface antigen virulence factors of E. coli K1 and group B Neisseria meningococcus and appears on the surface of mammalian neural cells. It's immunologic and binding properties was found to be temperature dependent. Various studies provided controversial conclusion on its conformational properties. Here we used circular dicronism to study the conformation of sialic acid in physiological solution.

The second most common enteric fever in Southeast Asia is S. paratyphi A which account ~15% of all the cases. As with S. typhi, S. paratyphi A is a habitant of and a pathogen for humans only and can be considered as a "clone." However, unlike S. typhi, paratyphi A does not contain a capsule. We have demonstrated that antiserum against its O-specific chain of LPS is bactericidal. The importance of the *O*-acetyl to the structure and immunologic properties of the O-specific polysaccharide was studied. We feel that the bactericidal antibodies elicited by our investigational O-specific polysaccharide-protein conjugate of S. paratyphi A is a clinical correlate of protection and clinical trials in endemic area are planned.

Escherichia coli *O*157 is considered as an "emerging pathogen." Disease caused by this organism exerts a high morbidity and mortality despite effective antimicrobials and supportive therapy. Incomplete data suggests that antimicrobial therapy of the hemorrhagic colitis caused by E. coli 0157 increases the incidence of the hemolytic uremic syndrome. The pathogenesis of this complication is unknown but it likely involves the action of the shigella-like toxins of this organism. We prepared 0157 O-specific polysaccharide protein conjugate prevention of infections caused by this pathogen. Vaccines prepared with different methods were evaluated in adult volunteers at the North Carolina Medical Center. The LDMI is also preparing conjugates using the shigella-like toxin as a carrier for E. coli 0157 O-specific polysaccharide conjugates in order to prepare therapeutic globulin for treatment of hemorrhagic colitis caused by E. coli 0157 and related organisms such as E. coli 0124.

A related pathogen, E. coli *O*111, is also an important cause of enteric infections including newborn nurseries. *O*111 strains of varying H types and with different virulence factors cause enteritis: this O type is found rarely in healthy individuals. Prevention by active immunization and treatment by passive immunization of this pathogen is planned. O-specific polysaccharide conjugates of this pathogen have been synthesized and found to induce bactericidal antibodies in mice. Due to the acid labile nature of colitose, a dideoxysugar in the O-specific polysaccharide, a non-denaturing process has been developed to detoxify the polysaccharide.

Vibrio cholerae 01 causes seven pandemic outbreaks in this century. Despite field trials involving millions in Bangladesh and Egypt, there is no satisfactory vaccines, either killed or attenuated strains. Furthermore, there is no concensus about what mechanism (s) confers protective immunity. There is strong evidence to show that serum vibriocidal antibodies confer protection and the majority antigenic component is the LPS. We propose that serum IgG against LPS can penetrate through the surface of intestine and inactive the inoculum. In addition to the common serotype Inaba 01, two other serotypes were emerging: one is the Ogawa of V. cholera 01 and the second one is a new non-01 V. cholerae strain 0139. Unlike 01, 0139 has a capsule. There is no cross-reaction between 01 and 0139. A vaccine against the LPS of 0139 has shown low vibriocidal activity. Conjugate vaccines based on the O-specific strain of the LPS of Ogawa and the capsular polysacchaide of 0139 were developed.

The only structural difference between serotypes Inaba and Ogawa was the terminal non-reducing end; Inaba has a hydroxy and Ogawa contains a methyl group at the C2 position. Several monoclonal antibodies of IgA and IgG were produced and showed different vibriocidal activities. We plan to study their specificities with synthetic oligosaccharides that mimic the O-chain to elucidate structural-immunological relations.

Lastly, this laboratory has re-investigated vaccine preparation against endotoxic shock caused by Gram-negative bacteria. Endotoxic shock is a major cause of nosocomial infection in U.S. and has the highest mortality rate. There is still no effective means for treatment and prevention. Most Gram-negatives share similar lipid A and core regions in their lipopolysaccharide. Therefore a vaccine that induces antibodies against these regions would cover a broad range of Gram-negatives. Both the core and the lipid A region are short and presented as poor immunogens. LDMI had prepared conjugates using hydrazine-treated LPS from E. coli J5, a rough mutant of E. coli 0111. Our results showed only species-specific protection against E. coli in animals. We plan to study new methods for conjugating short polysaccharides to carrier proteins.

Diphtheria mutants such as cream 197 or cream 9 have reduced toxicity and, unexpectly, also have lower immunogenicity. Various mutants on both A and B subunits had been prepared to reduce the toxicity even further and, at the same time, retain the immunogenicity. The protein conformation analysis can provide a way to monitor such selection process in addition to biological assays. Our laboratory is interested in studying the conformational analysis of these mutant proteins and use them as carrier protein for our conjugates.

Methods Employed:

High performance ion exchange chromatography (HPAEC) was used to detect acid.

Conformation energy calculations were performed with Program Random Molecular Mechanics (RAMM) and molecular dynamics simulations was done in the Consistent Valence Force Field (CVFF). Protein and saccharide conformations were analyzed with circular dichroism (Jasco 700). Binding was measured by titration.

Vi and the O-specific polysaccharides were purified and derivatized by methods devised or used by the LDMI. Polysaccharides were studied by NMR, SDS PAGE for purity, Hestrin reaction for O-acetyl and 2-nitro-phenyl hydrazine for carboxyl groups, immunodiffusion and bioassays for antigenicity. Carrier proteins were purified and their purity and safety assayed by physical chemical methods. Conjugates of these polysaccharides and proteins were synthesized by methods devised at the LDMI and prepared under good manufacturing practices to achieve conditions suitable for FDA approval and clinical use.

The new method for Vi conjugation was to derivatize the carrier protein with adipic dihydrazide and then bound to Vi in the presence of water soluble carbodiimide. The new method for paratyphi A conjugation was to activate polysaccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) at pH 7 to 8 with or without adipic dihydrazide: the former will form a conjugate directly bound to the protein.

Oligosaccharides of cholera Inaba or Ogawa were synthesized by Dr. P. Kovac and staff (LMC, NIDDK). Diphtheria mutant proteins were prepared and purified by Dr. G. Johnson (DBP, CBER). Evaluation of the experimental vaccines in mice used clinically relevant protocols designed by the LDMI. Serum antibody responses induced in humans was assayed by quantitative and standardized ELISA. Functional assays, including the vibriocidal assay for V. cholerae, bactericidal assay for E. coli 0157, S. paratyphi using in vitro methods. Determination of the safety of these conjugates used methods outlined in the U.S. CFR. Clinical studies in humans were enacted after approval by the appropriate NIH, FDA and local IRB. Oligosaccharide binding with V. cholera monoclonal antibodies were measured either by fluorescence titration or by ELISA inhibition.

Major Findings:

In collaboration with the Vietnam Public Health Department of the Institute Pasteur, Ho Chi Ming City, a double blinded clinical trial phase II using Vi conjugate synthesized with SPDP as a linker was injected into 108 children 5-15 years old and 110 children 2-4 years old in Dong Thap Province. There were no side reactions. Preliminary serological data showed more than 90% vaccinees have 4-fold rise Vi antibodies. Children between 2-4 years of age receiving booster does (two injections, six weeks apart) showed a booster rise in their Vi antibody levels.

We improved our synthesis of Vi conjugates. The improved method uses ADH derivatized protein and elicited higher antibody levels in mice than the conjugates synthesized previously with SPDP. The new method eliminated possible cross-linking of Vi and therefore reduced the molecular size of the conjugate. The improved technology increases the yield and reproducibility of the conjugate procedure. A clinical lot was prepared and phase I study planned in teenage children in Vietnam. New ELISA assay was established in our laboratory for determination and standardization of serum Vi antibodies in replace of RIA.

O-specific polysaccharide from S. paratyphi A was conjugated with tetanus toxoid by two different linking methods: a) activating and derivatizing polysaccharide with CDAP and ADH then linked to a protein; and b) activating polysaccharide with CDAP only then linked directly to a protein. These conjugates showed similar immunogenicity in mice. Two vaccine lots were prepared and phase I clinical trials were conducted with the Vaccine Institute of Vietnam and Pasteur Institute at Dong Thap Province. In phase I, 18 adults were injected with one of the two vaccines. There were no side reactions and 78% vaccinees had a 4-fold rise in their serum LPS antibodies. Subsequently, 96 teenage children, age 13-17 were injected with one of the vaccines. The immune response in this age group is similiar to that in adults. Both groups showed that vaccine synthesized with method (b) had higher antibody levels.

Four monoclonal antibodies, two IgG and two IgA, against V. cholera Ogawa or both Inaba and Ogawa, were tested for their binding activity with synthetic LPS O-polysaccharides. We found that, for Ogawa serotype, as small as monosaccharide, there is strong binding ($Ka=10^5$) with Ogawa monoclonal antibodies. In contrast, both Inaba or Ogawa oligosaccharide failed to inhibit the binding of LPS with the monoclonal antibodies. This data indicates that the specificity of the Ogawa monoclonal antibodies are probably against the non-reducing of the LPS and has a cavity shaped binding site. The vibrioidal titer of the Ogawa monoclonal IgG is 10^2 higher than monoclonal IgG against Inaba. Based on these and other studies, we are in the process of synthesizing O-specific polysaccharide up to

12 units which is comparable with the native LPS length. Methods using linkers such as squric acid or polylysing to conjugate with protein had been tested and preliminary results showed that the antigenicity of the polysaccharides has not changed. The other serotype of V. cholerae that causes disease, 0139, has a capsule. We have completed in purifying this polysaccharide with little contamination from its LPS.

The O-specific polysaccharide of E. coli 0157 LPS was detoxified with acetic acide or hydrazine and derivatized with adipic acid dihydrazide and bound to P. aeruginosa recombinant exotoxin A. In collaboration with Dr. J.C. Parke, Jr., 87 healthy volunteers from the Carolinas Medical Center were injected with one of the conjugate. All three conjugates elicited serum antibodies against LPS (>100 fold rise) with bactericidal activity to the 0157 LPS. The highest rise of LPS antibodies was of the IgG class. The antibody levels declined slightly (to ~80 fold rise) after six months.

In collaboration with Dr. Y.C. Lee, Johns Hopkins University, using smooth LPS from E. coli 0157 and rough mutant from S. minnesota 585 as a model, the condition for hydrolys's were studied by monitor the temperature and pH. 60° was found to be the highest temperature at 17% acetic acid that the acid still intacted. Higher temperature or stronger acid will also reduce the concentration of phosphate group in the lipid A region.

Conformational analysis of sialic acid and its derivative were studied with circular dichroism and intrinsic viscosity. Our results showed that there is no preferred helical structure in the native or de-N-acetylated sialic acid. Binding with multivalent ions did not induce conformational transition either. Our data indicated that the binding of sialic acid to its antibodies, may not contain a specific conformationally controlled determinant, as has been put forth. Rather, the data suggest that more than nine saccharride units might be required for a cooperative process upon binding, which balances the enthalpy of association and the unfavorable change of entropy. In collaboration with Dr. G. Johnson, several mutants of diphtheria toxin A subunit (the enzymatic unit) were prepared and their conformations analyzed by CD. The results showed that mutant changing histi to glycine at position 21 had the closest conformation as the wild type. This mutant (H21G) had reduced toxicity but retained its immunogenicity. The second most similar mutant is changing glycine 79 to aspartic acid at position 21. This mutant had reduced toxicity as well as immunogenicity compared with the wild type. The latter property made it not suitable for vaccine usage.

Proposed Course:

We plan to choose one of the better Vi conjugates vaccines synthesize by two different methods for the phase III field trial in areas near Dong Thap Province, Vietnam, for children 2-5 years. At the same time a safety and immunogenicity trial will be conducted for infants from 3 month the 2 years old. The S. paratyphi A vaccine synthesize without a linker will be injected to younger children 2-4 years old with either one or two injections. After completion of this phase II study, S. paratyphi A conjugate will be the control vaccine for the field trial of Vi conjugate. Approximately 8000 children between 2-5 years of age will be injection with one of the conjugate and observed 2-3 years.

Trials of the 0157 conjugates in children in areas such as Seattle and Japan are planned in order to evaluate the potential for these vaccines to stop outbreaks and to eliminate carriage of E. coli 0157. A phase II studied is planned with the Children's Hospital at Seattle for 2-years-old children. Plasmaphoresis of sera from high responders in adult volunteers are planned for therapeutic usage of this vaccine. The preparation of conjugates using non-toxic shigella-like toxins I and II, subunit B for therapeutic globulin as a treatment of hemorrhagic colitis caused by E. coli 0157 is planned. Since cattle are carriers of this organism, in collaboration with Dr. A. Donahue-Rolfe, Tufts University, vaccine for cattle will be studied to eliminate)157 from its reservior.

V. cholera vaccines against Inaba and Ogawa will be prepared by conjugate with proteins with O-specific side-chain. Both the native and synthetic O-specific polysaccharide will be used as starting material and results will be compared. The difference between these two preparation will be that the synthetic material does not contain the core region sugars. Vaccines against 0139 will be prepared. The immunodominant sites and its structure will be studied in detail for better understanding of this new emerging disease.

Vaccines for endotoxic shock will be prepared from two mutant strains: J5 from E. coli and S. minnesota R595. At least two different methods for conjugation of these oligosaccharides will be tested. One will utilize the carboxylic on the KDO residue on the core. The other will be to remove the *N*-acyl linked lipid with hydrazine and then link residual disaccharide region of the lipid A to polyacrylamide as a backbone. Both methods should preserve the diphosphate region of the lipid A which is believed to be immunodominant.

Significance to Biomedical Research and the Program of the Institute:

Conjugate vaccine for typhoid fever and S. paratyphi A have shown to be immunogencies and induced booster effect in children 2-4 in Vietnam. The field trial in this age group will provide future opportunity to license these conjugate vaccine. Prevention of enteric bacterial infections, especially of infants and children, will be an important achievement of the NICHD. The development of immunotherapy against the complications of E. coli 0157, 0111 as well as other enteroinvasive pathogens, may establish a new approach for treating these serious disease caused by an emerging pathogen.

The major cause of nosocomial death is the endotoxic shock caused by Gram-negative organisms. There is yet no available treatment for this serious infection. From our earlier study and clinical trials of monoclonal antibody study in other laboratories, the antibodies against the diphosphate di-glucosamine region will be protective. Both the prevention and therapeutic approach are taken here.

Synthetic cholera O-polysaccharide to prepare for conjugate vaccine will lay down a new era for polysaccharide vaccine and simplify procedures to monitor the quality of the vaccine.

Polysaccharide chemistry is an important area in development of conjugate vaccines. New techniques to analyze the structure, conformation and function of each group will provide us new insight on their relation to immunogenicity.

Publications:

Liao J, Nickerson KG, Bystricky S, Robbins JB, Schneerson R, Szu SC, Kabat EA. Characterization of a human monoclonal IgM antibody (IgMBEN) specific for the Vi capsular polysaccharide of Salmonella typhi. Infect Immun 1995; 63:4429-32.

Szu SC, Gupta R, Kocac P, Taylor DN, Robbins JB. Development of O-specific polysaccharide-protein conjugates is based upon the protective effect of serum vibriocidal antibodies against cholera. Bull Inst Pasteur 1995; 93:223-318.

Konadu E, Shiloach J, Bryla DA, Robbins JB, Szu SC. Synthesis, characterization and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of Salmonella paratyphi A bound to tetanus toxoid, with emphasis on the role of O-acetyls. Infect Immun 1996; 64:2709-15.

Bystricky S, Pavliak V, Szu SC. Characterization of colominic acid (poly a(2-8)-D-NeupNAc by circular dichroism and viscosity analysis. Biophys Chem (accepted for publication).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01311-03 LDMI

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A Polysaccharide Vaccine for Mycobacterium tuberculosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.B. Robbins	Chief	LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)*

LCDB, NIDDK (J. Shiloach); CBER, FDA (S. Morris, J. Muller); Pasteur-Mérieux Serum et Vaccins, Lyon, France (D. Schulz).

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.5	4.3	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tuberculosis remains a serious and common disease worldwide. In the U.S. emergence of multi-antibiotic resistant strains, due to the rapid passage of Mycobacterium tuberculosis in patients with AIDS, now poses a public health problem. Although the most frequently used vaccine in the world, there is no scientifically-based evidence that BCG prevents primary pulmonary tuberculosis and its protective effect in children has not been related with a protective antigen or a host immune component. Our research into a new vaccine for tuberculosis is based upon the similarity of primary infection caused by M. tuberculosis with that of capsulated bacterial respiratory pathogens, viz: 1) epidemiologic data which show that tuberculous meningitis has a similar age distribution as capsulated bacteria; 2) the presence of a capsular polysaccharide on M. tuberculosis and other mycobacteria in vitro and in vivo; and 3) that BCG and protein components of this and wild-type strains have prolonged but never conferred protection against challenge with wild-type M. tuberculosis in animals. This $\alpha(1\rightarrow2)$ glucose moiety is identified in extracts of M. tuberculosis, but not in BCG, by its reactivity with pneumococcal type 12F typing antisera. Mycobacteria were extracted with saline and then with 1% Triton. The residual organisms are extracted by the hot phenol method used for LPS of Gram-negatives. The resultant product precipitates with pneumococcal type 12F antisera giving an identity reaction with dextran 1299 which has kojibiose residues. Animal and human antisera, reactive with pneumococcus type 12 and dextran 1299 elicit complement-dependent killing of M. tuberculosis strains Erdmann and wild-type M123 in vitro. The cidal reaction is inhibited by a synthetic kojipentaose but not by an $\alpha(1\rightarrow4)$-D-Gal trimer. Conjugates have been prepared with kojipentaose and by another surface glucan of M. tuberculosis.

PHS 6040 (Rev. 5/92)

Others:	R. Schneerson	Research Medical Officer	LDMI, NICHD
	V. Pozsgay	Visiting Scientist	LDMI, NICHD
	Z. Dai	Visiting Fellow	LDMI, NICHD
	E. Dubois	Visiting Fellow	LDMI, NICHD
	N.W. Tolson	Biologist	LDMI, NICHD

Project Description:

Despite its discovery over one century ago, there is yet no scientifically-based evidence for the protective effect of BCG or any other vaccine against of primary pulmonary tuberculosis. Tuberculosis continues to be a major cause of morbidity and mortality throughout the world and, in the U.S., multi antibiotic resistant strains of *M. tuberculosis* pose an important public health problem.

Administration of BCG in infants prevents tuberculous meningitis in young children and its administration into the bladder is an effective therapy for bladder carcinoma. But the protective antigen and the host immune response elicited by BCG that are correlated with these protective actions are not characterized. The skin test for tuberculosis infection, detected by PPD or a comparable product, is mediated by T-cells but, is not related to protective immunity to tuberculosis.

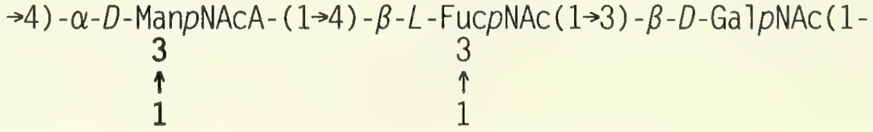
There is experimental states that serum antibodies should be reinvestigated as a *protective* immune mechanism to tuberculosis, *viz*: 1) the age incidence of tuberculous meningitis, as an example of a systemic complication of a respiratory infection, is similar to other capsulated bacterial pathogens; 2) similar to other capsulated pathogens, polymorphonuclear white blood cells are the first to gather at the site of an inoculum of *M. tuberculosis*. Although WBC engulf and kill some organisms, these cells are replaced with mononuclear cells after 24 hours 3) *M. tuberculosis* and other pathoenic mycobacteria are surrounded by a clear zone, referred to as the "ETZ", shown to be a polysaccharide; and 4) preliminary evidence that serum antibodies, induced in some rabbits by multiple intravenous injections of inactivated mycobacteria, were correlated with protein against challenge. One polysaccharide, identified as Polysaccharide II by its discoverer, Dr. Florence Seibert, seems to be a reasonable candidate to investigate as a protective antigen.
Another cell component is also being investigated is the so-called "glycogen" of *M. tuberculosis*. Surprisingly, bacterial glycogen is antigenic and elicits antibodies that have opsonphyagocytosis activity.

Objectives:

To establish an *in vitro* sopsonphagocytosis and killing assay for *M. tubercuosis* with human antibodies, complement and white blood cells. To isolate the "Polysaccharide II" fraction of *M. tuberculosis* and to confirm its structre by modern techniques. To synthesize oligosaccharides of $\alpha(1\rightarrow 2)$-*D*-glucose and to characterize their immunochemical and biological properties upon opsonphagocytosis of *M. tuberculosis*. To synthesis poly or $(1\rightarrow 2)$ *D*-glucose conjugate and assay opsonphagocytosis activity of immune sera.

Methods Employed:

Pneumococcal type 12F polysaccharide, a component of the 23 valent vaccine contains an $\alpha(1\rightarrow 2)$-*D*-glucobiose moiety:



$$\rightarrow 4)\text{-}\underset{\substack{3\\ \uparrow\\ 1}}{\alpha\text{-}D\text{-Man}p\text{NAcA}}\text{-}(1\rightarrow 4)\text{-}\underset{\substack{3\\ \uparrow\\ 1}}{\beta\text{-}L\text{-Fuc}p\text{NAc}}(1\rightarrow 3)\text{-}\beta\text{-}D\text{-Gal}p\text{NAc}(1\text{-}$$

α-D-Gl*cp*(1→2)-*α-D*-Glc   *α-D*-Gal

Streptocococcus pneumoniae type 12F (Leontein, et al., 1981).

Typing antisera to this pneumococcal type was used to identify this unusual glucan in dextrans: one dextran denoted as 1299, in particular, has been studied extensively because of its unusual reactivity with type 12 pneumococcal antiserum. Either rabbit or horse pneumococcal type 12F antiserum has been used to detect the α(1→2)-D-glucobiose moiety by double immunodiffusion. Curiously, two courses of multiple intravenous injections of formalin-treated M. tuberculosis, strain H37R, failed to elicit antibodies to pneumococcus type 12F or to dextran 1299. Immune sera from these animals, however, precipitated with another polysacchride from M. tuberculosis known as lipoarabinomannan.

An in vitro opsonophagocytosis assay for M. tuberculosis, copied after that system used for assaying bactericidal antibodies to Group B streptococci. Animal typing antisera, murine monoclonal antibodies reactive with dextrans and those elicited by immunization of mice with tubercle bacilli were evaluated for their reactivity with the α(1→2)-D-glucose moiety. The surface polysaccharides of several M. tuberculosis strains, isolated from patients were extracted sequencially with saline and then with 1% triton X-100. The polysaccharide containing materials were passed through an anion-exchanger and the neutral macromolecules passed through sephacryl S-300. The latter resolved 2 major peaks and 1 minor peak all composed of carbohydrate. The structure of these materials and their immunologic properties as protein conjugates are under investigation.

Electron microscopy of M. tuberculosis incubated with pneumococcal type 12F antisera showed intense surface strain no observed with murine antibodies to a surface protein.

Human antibodies to pneumococcal type 12 were purified from the plasmapheresis of an adult vaccination previously with a pneumococcal tpe 12F-diphtheria toxoid conjugate. Purified human antibodies were prepared by affinity chromatography with a type 12F polysaccharide-4B amino-hexyl Sepharose matrix.

Major Findings:

A saccharide-like structure, reactive with the anti-α(1→2)-D-glucose moiety of rabbit typing, was purified from M. tuberculosis strain M123 from a patient diagnosed with pulmonary tuberculosis. The preliminary isolation was done with strains grown on Long's media at CBER, FDA and at the Pasteur-Mérieux Institut. Extraction was accomplished by the hot-phenol method for preparing LPS from enterbacteriae. The α(1→2)-D-glucose was more definitely established by gas-chromatography and mass spectroscopy of methylated derivative of the saccharide.

Dextran 1299, conjugated to chicken serum abumin, even when injected along with complete Freund's adjuvant in mice, did not elicit anti-α(1→2)-D-glucose. Mice so immunized elicited precipitating antibodies to the α(1→4) and α(1→3)-D-glucose bonds of other dextrans.

Rabbit, horse and human anti-pneumococcus type 12 antibodies killed M. tuberculosis strains Erdmann and M123 in the presence of complement and human

WBCs.

The antibody mediated, complement-dependent killing was inhibited by a kojipentaose but, not with an $\alpha(1 \rightarrow 4)$-D-Gal-trimer.

Significance to Biomedical Research and the Program of the Institute:

The development of a vaccine based upon induction of serum antibodies to a surface polysaccharide of *M. tuberculosis* would provide an important new public health measure as well as a conceptual tool for understanding immunity to mycobacteria.

Publications:

Dai Z, Morris S, Muller J, Schulz D, Pozsgay V, Shiloach J, Schneerson R, Robbins JB. Serum a(1->2)-D-glucan antibodies initiate complement-dependent opsonophagocytic killing of *Mycobacterium tuberculosis* by human blood neutrophils. Proc Natl Acad Sci USA (accepted for publication).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01312-02 LDMI

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation and Purification of Subunit B of Shiga Toxin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	V. Pozsgay	Visiting Scientist	LDMI, NICHD
Others:	J.B. Robbins	Chief	LDMI, NICHD
	S.C. Szu	Research Chemist	LDMI, NICHD
	L.B. Trinh	Chemist	LDMI, NICHD

COOPERATING UNITS *(if any)* LCDB, NIDDK (J. Shiloach); Tufts University School of Veterinary Medicine, N. Grafton, MA (A. Donahue-Rolfe); Massachusetts General Hospital, Boston, MA (S.B. Calderwood)

LAB/BRANCH Laboratory of Developmental and Molecular Immunity

SECTION Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS: 0.3	PROFESSIONAL: 0.1	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Shiga toxins are multimeric proteins consisting of a single enzymatic subunit (subunit A, mw 32 kDa) and a symmetrically arranged, pentameric subunit B (mw 7.7 kDa). The latter mediates the binding of the holotoxin to cell-surface glycolipids as the first step of its endocytosis. Galabiose is the minimum binding receptor that recognizes the toxin, placed either terminally or internally in glycolipids. The binding is stronger with P blood group determinant trisaccharides, such as the P_k antigen and the P_1 antigen. Specific adsorbents of Shiga (Shiga-like) toxins may aid the development of serologic tests for rapid diagnosis of Escherichia coli infections, and receptor-analog oligosaccharides that inhibit the binding of these toxins to their cell-surface receptors can be candidates for therapeutics against such diseases. The non-toxic subunit B may be a candidate as a carrier in the synthesis of polysaccharide-protein conjugates. Based on the reported carbohydrate-specificities of subunit B, we developed a simple and efficient method for the preparative scale isolation of this toxin fragment, using a receptor-analog affinity sorbent. A P_1 antigenic trisaccharide, equipped with an anchor at its reducing end, was covalently attached to a solid support through this anchor. The resulting affinity material was used over twenty cycles to isolate subunit B of Shiga toxin from the fermentation fluid of a non-virulet Vibrio cholera strain that contained the gene for this protein, in a semi-automated fashion. The resultant B-subunit was covalently bound to the O-specific polysaccharide of E. coli O-157 and the resultant conjugate shown to induce LPS and antitoxin antibodies in mice.

Project Description:

The proper selection of the carrier protein in the design of carbohydrate-protein conjugates is crucially important and the search for proteins that can be alternative to those already established continues. Non-toxic subunits of bacterial toxins may have the potential of being immunogenic carriers and subunit B of the Shiga and Shiga-like family of secreted toxins can be a candidate for this purpose. The gene of this subunit has been expressed in a Vibrio cholera strain which lacks the gene for subunit A that is responsible for the toxicity of the holotoxin. The carbohydrate specificity of subunit B has been established and a procedure was reported for the isolation of this subunit using receptor-analog affinity chromatography. The very low amount of the carbohydrate hapten in the glycoprotein from the hydatid fluid that was used in the published protocol precludes scale-up. Also, the method may be objectionable as the subunit to be purified is intended for human and animal experiments. Our goal was to design a method for the isolation and purification of subunit B of Shiga toxin.

Objectives:

To synthesize a receptor-analog affinity sorbent for the preparative scale isolation of subunit B of Shiga toxin and to use it in a semi-automated fashion to recover this protein from the fermentation fluid of V. cholera (Strain 0395-N1).

Methods Employed:

The P_1 trisaccharide containing D-galactose and N-acetyl-D-glucosamine residues

α-D-Gal$\underline{p}$-(1→4)-β-D-Gal$\underline{p}$-(1→4)-β-D-Glc$\underline{p}$NAc

was assembled from suitably protected monosaccharides in stereoselective manner, using thioglycosides and 2-(trimethylsilyl)ethyl glycosides as major intermediates. The trisaccharide was attached to a heterobifunctional spacer that contained a reactive, aldehyde group at its terminus. Through this group the hapten-spacer assembly was coupled to a hydrazino group-containing solid support, using the method of reductive amination.

Subunit B of Shiga toxin was produced by Dr. J. Shiloach (LCDB, NIDDK) by fermentation of a non-virulent V. cholera strain (0395-N1) obtained from Drs. A. Donahue-Rolfe and S.B. Calderwood (Tufts University School of Veterinary Medicine and Massachusetts General Hospital). The periplasmic extract was loaded directly on the affinity column from which subunit B could be eluted selectively in high purity. In each cycle approximately 10 mg of pure subunit B was obtained. Until now the column was used over twenty cycles with unchanged capacity. The identity and purity of subunit B so prepared was assessed by SDS-PAGE electrophoresis and its antigenicity was demonstrated by its reaction with a monoclonal antibody directed against subunit B.

Major Findings:

We established that subunit B of Shiga toxin can be isolated from the fermentation fluid of a non-virulent V. cholera strain containing the gene for subunit B in high purity using a synthetic trisaccharide-based affinity material.

Proposed Course:

The potentials of subunit B of Shiga toxin as a carrier in polysaccharide-protein conjugates will be evaluated.

Significance to Biomedical Research and the Program of the Institute:

The availability of novel protein carriers is important for the development of carbohydrate-protein conjugate vaccines of improved efficacy.

Publications:

Pozsgay V, Trinh L, Shiloach J, Robbins JB, Donahue-Rolfe A, Calderwood S. Purification of subunit B of Shiga toxin using a synthetic trisaccharide-based affinity matrix. Bioconjug Chem 1996; 7:45-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01313-02 LDMI

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synthetic Vaccine Against Shigellosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	V. Pozsgay	Visiting Scientist	LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)*

LMC, NIDDK (C.P.J. Glaudemans); National Institute of Standards and Technology, Gaithersburg, MD (B. Coxon)

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Shigellosis caused by Shigella dysenteriae type 1 continues to be a major enteric disease worldwide and most strains are now antibiotic resistant. Although the need for vaccines to control this disease has been documented by the WHO, there is no licenced vaccine against shigellosis. Following the discovery by Robbins and coworkers that serum antibodies against the O-specific polysaccharide (OSP) of Shigella sonnei immunity to disease caused by this pathogen in humans, we hypothesized that extended fragments of the OSP may also be suitable for the induction of protective antibodies when coupled to immunogenic proteins, provided that the conformational ensemble of such saccharides approaches that of the conformational determinant of the native polysaccharide. The use of synthetic saccharides of defined structure instead of native polysaccharides of complex architecture is likely to offer advantages including enhanced uniformity of conjugates and elimination of the analytical difficulties associated with the established, polysaccharide-protein vaccines. Based on this hypothesis we are developing synthetic oligo- and poly-saccharide-based immunogens of well-defined characteristics. We designed a strategy to prepare fragments of the OSP of S. dysenteriae type 1. The OSP consists of a tetrasaccharide repeating unit that is composed of D-galactose, N-acetyl-D-glucosamine, and L-rhamnose. Starting from monosaccharide building blocks that carry orthogonal protecting and activating groups, a tetrasaccharide donor/acceptor molecule was assembled. Iterative combination of this building block afforded di-, tri- and tetramers of the repeating unit corresponding to octa-, dodeca- and hexadeca-saccharides. The dodeca- and the hexadeca-saccharides exhibit a high degree of conformational similarity to the native O-SP by NMR. The first conjugate of a tetra-repeating unit has been syntheiszed. In vitro experiments showed that the synthetic saccharides inhibit the binding of the OSP to homologous monoclonal antibodies.

PHS 6040 (Rev. 5/92)

Others:	J.B. Robbins	Chief	LDMI, NICHD
	R. Schneerson	Research Medical Officer	LDMI, NICHD
	A.B. Karpas	Microbiologist	LDMI, NICHD
	C.W. Broome	Chemist	LDMI, NICHD

Project Description:

Shigella dysenteriae type 1 is the causative organism of epidemic and endemic diarrhea and dysentery in may parts of the world with high mortality and morbidity. It is dysentery (fever, mucous and blood in the stool) which is the leading cause of growth retardation in the word. An approach to combat this disease can be vaccine development. In spite of the fact, that the causative organisms have been identified a century ago, there is no licenced vaccine against shigellosis. The need for such vaccines is documented by a World Health Organization document which accords priority to the development of anti-shigellosis vaccines. Although shigellosis can be induced in animals, the animal model for shigellosis has limitations which is highlighted by the fact, that the challenge dose for monkeys is 10^{11} organisms which is 10^{8} times higher than the dose required to induce shigellosis in healthy humans. An essential virulence factor of Shigellae strains is the O-specific polysaccharide component of their respective lipopolysaccharides. Only smooth strains, having fully expressed O-SPs are virulent, whereas rough strains that are devoid of these structures, are not. The essential role of the O-specific polysaccharides of Shigellae and also other enteropathogenic bacteria led to the hypothesis of Robbins and co-workers, that serum IgG antibodies to the OSPs confer protective immunity to the host. The validity of this hypothesis was substantiated by experiments with conjugate vaccines containing purified, O-specific polysaccharides covalently linked to an immunogenic protein. Evidence indicates, that anti-O-specific polysaccharide antibodies in vivo interact with the lipopolysaccharides on the bacterial cell surface. However, little is known about the mechanism of such interactions and about the underlying factors at the molecular level, such as the importance of the hapten geometry, and hapten density in our current conjugate vaccines, concerning either antibody production or antibody recognition.

Objectives:

We surmise that extended fragments of the O-specific polysaccharides may be suitable for the induction of protective antibodies when coupled to immunogenic proteins, provided that the conformational ensemble of such saccharides approaches that of the conformational determinant of the native polysaccharide. Therefore we have been studying chemical synthetic approaches to such saccharides. We are also studying the conformational properties of the synthetic oligosaccharides in relation to the conformation of the native O-specific polysaccharide. A scheme has been devised to covalently attach the saccharides to proteins. The evaluation of the immunologic properties of the synthetic oligosaccharides up to a hexadecasaccharide is currently under study.

Methods Employed:

The O-polysaccharide of S. dysenteriae type 1 consists of the linear tetrasaccharide repeating unit, containing α-linked N-acetyl-D-glucosamine, D-galactose and L-rhamnose as the monosaccharide constituents.

$[3)\text{-}\alpha\text{-L-Rha}\underline{p}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Gal}\underline{p}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}\underline{p}\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}\underline{p}\text{-}(1\rightarrow]_n$

Our plan was to construct a repeating unit block that can serve both as a glycosyl donor and a glycosyl acceptor in a stepwise fashion from monosaccharide synthons and use it in an iterative manner. A key tetrasaccharide intermediate was selected for this purpose. An important feature of this building block is that after its initial coupling with the aglycon under mild conditions that do not effect the functional/protecting groups, the site of the chain elongation can be unmasked by a minimal protecting group manipulation involving the chemoselective removal of one only protecting group. The major role of the aglycon is to allow one-point, covalent attachment of the synthetic saccharide constructs to proteins. Moreover, the aglycon (spacer) provides the necessary distance of the saccharide chain from the protein. The aglycon can also be exploited fro polymerization of the saccharides into fully synthetic, comblike macromolecules. The monosaccharide synthons were prepared from D-galactose, D-mannose and L-rhamnose, using the methods of synthetic organic chemistry and were combined in stereoselective, stepwise manner. The structures of all the synthetic intermediates and the target saccharides **1-4** were confirmed by analytical and spectroscopic methods including various mass spectroscopic protocols and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

$[3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow]_n\text{-O}(\text{CH}^2)^5\text{CONHNH}^2$

1 n=1
2 n=2
3 n=3
4 n=4

Particular support was provided by the ^{13}C NMR spectra of the dodeca-(**3**) and hexadeca-saccharides (**4**) in the latter of which the anomeric resonances for the four Gal residues, 3 and 4 Rha residues, and 3 GlcNAc residues, respectively, coincide with the corresponding resonances of the native, O-polysaccharide of *S. dysenteriae* 1. This coincidence not only establishes the stereochemical integrity of the interglycosidic linkages but also indicates that compounds **3** and **4** express a high degree of conformational similarity to the native polysaccharide. A new heterobifunctional linker containing a free caroxyl group and a masked aldehydo moiety was developed and used for the covalent attachment of the synthetic saccharides to human serum albumin at multiple sites on the protein. Briefly, the amino group installed at the terminus of the aglycon of the synthetic saccharides was acylated with the spacer moiety using carbodiimide- or N-hydroxysuccinimide-type activation. Next the protecting groups of the aldehydo function of the linker moiety were removed by mild acid hydrolysis. Finally, the saccharide-spacer construct was anchored to the protein by reductive amination. The incorporation of saccharides into the protein was determined by rigorous, mass spectroscopic analysis of the simi-synthetic glycoconjugates, using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. For example, the hexadecasaccharide-HSA conjugate contains an average of 11 saccharide chains per HSA molecule, that is the carbohydrate content of the glycoconjugate is approximately 25%. Similar incorporation levels were achieved for the tetra-, octa-, and dodeca-saccharide haptens.

Major Findings:

We established that preparative-scale quantities of extended fragments of the native O-specific polysaccharide of *S. dysenteriae* type 1 can be synthesized by iterative combination of a tetrasaccharide building block. Nuclear magnetic resonance studies indicate that an internal portion of the octasaccharide fragment, corresponding to two contiguous repeating units, already expresses conformational similarity to the native O-specific polysaccharide. A higher degree of conformational similarity is expressed by the dodeca- and the hexadeca-saccharides which correspond to three and four repeating units, respectively. Preliminary experiments indicate that the synthetic saccharides inhibit the binding of O-specific polysaccharide specific monoclonal antibodies raised against heat-killed S. dysenteriae type 1. The semi-synthetic neoglycoconjugates prepared by covalent attachment of the synthetic saccharides through a spacer moiety to human serum albumin are antigenic against anti-Shigella dysenteriae type 1-specific antibodies as determined by immunodifuusion experiments.

Proposed Course:

The antigenic properties of the saccharide fragments of the O-specific polysaccharide of S. dysenteriae type 1 will be evaluated using several O-specific polysaccharide-specific monoclonal antibodies. The efficacy of the covalent conjugates of the synthetic saccharides and immunogenic proteins. to induce anti O-specific polysaccharide antibodies will be studied. The protective effect of these antibodies will be evaluated. The capacity of the synthetic conjugates to induce antibody will be correlated to their molecular characteristics including hapten size and hapten density.

Significance to Biomedical Research and the Program of the Institute:

Shigellosis is a continuing public health problem in both the developing and industrialized countries and the development of an effective vaccine will be an important step toward eliminating this disease.

Publications:

Pozsgay V, Coxon B. Stereoselective preparation of alkyl glycosides of 2-acetamido-2-deoxy-α-D-glucopyranose by non-classical halide-ion catalysis and synthesis and NMR spectroscopy of α-D-Galp-(1→3)-α-D-GlcpNAc-OMe. Carbohydr Res 1995; 177:171-78.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01314-02 LDMI

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis and Synthesis of Carbohydrate Antigens of Mycobacterium tuberculosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	V. Pozsgay	Visiting Scientist	LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)*

LAC, NIDDK (N. Whittaker); National Institute of Standards and Technology, Gaithersburg, MD (B. Coxon)

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mycobacterium tuberculosis, the causative organism of tuberculosis is responsible for approximately three million death cases annually and the mortality rate of infections caused by multiple drug-resistant strains of this bacterium exceed 80%. M. tuberculosis is coated by a number of polysaccharides of unusual structure. A unique component termed polysaccharide II is composed of alpha, 1,2-linked D-glucose residues. Schemes were designed to isolate this polysaccharide from extracts of M. tuberculosis. We demonstrated that the major monosaccharide components in soluble polysaccharide fractions from Mycobacteria are arabinose, mannose and galactose. A polysaccharide fraction was also identified which contains mannose and glucose in approximately equal amounts. We demonstrated the presence of a glycogen-like polysaccharide as one of the extracellular polysaccharides of Mycobacteria and developed a method for the chemical synthesis of oligosaccharides corresponding to the putative polysaccharide II.

PHS 6040 (Rev. 5/92)

Others:	J.B. Robbins	Chief	LDMI, NICHD
	R. Schneerson	Research Medical Officer	LDMI, NICHD
	E. Dubois	Visiting Fellow	LDMI, NICHD
	C.W. Broome	Chemist	LDMI, NICHD

Project Description:

Tuberculosis caused by Mycobacterium tuberculosis continues to be a major cause of death worldwide and the fatility rate of this disease when caused by multiple antibiotic-resistant strains in immunocompromised patients is higher than 80%. There is increasing evidence that antibodies against mycobacterial surface antigens may play a role in protection.

The major component of the extracellular fraction of M. tuberculosis termed polysaccharide II by Siebert was shown to be a linear of α1,2-linked D-glucose residues. Experiments were designed to isolate and identify this unique polysaccharide for studies of its possible role as a protective antigen. As an alternative approach to this polysaccharide, chemical synthetic approaches were also planned.

Objectives:

To identify glucose-containing polysaccharide(s) among the carbohydrate fractions of the cell-surface polysaccharides of M. tuberculosis. To synthesize oligosaccharides containing α1,2-linked D-glucose residues, corresponding to polysaccharide II and to covalently attach them to high molecular weight carriers.

Methods Employed:

(a) Polysaccharide fractions of M. tuberculosis were analyzed by a combination of chemical and instrumental methods. These included chemical conversion of the saccharides into (partially methylated) alditol acetates which were analyzed by gas chromatography combined with mass spectroscopy. The intact saccharide fractions were also analyzed by high-resolution nuclear magnetic resonance spectroscopy.

(b) The putative glucan antigen of M. tuberculosis termed Polysaccharide II is a linear polysaccharide containing α-(1→2)-linked D-glucopyranose residues. Retrosynthetic analysis of this polysaccharide indicated that a single building block, equipped with a temporary, non-participating group at O-2 may be sufficient for its construction. We have chosen the glucosyl donor 3,4,6-tri-O-acetyl-2-O-benzyl-β-D-glucopyranosyl bromide for this purpose which was obtained stereoselectively from the corresponding, α-phenylthio glucopyranoside. The donor afforded α-linked glucosides upon solvolysis with primary alcohols in a completely stereoselective manner. Subsequent, iterative chain extension involving chemoselective liberation of the elongation site followed by silver salt-catalyzed glucosidation with the donor/acceptor building block afforded fully protected kojibiose to kojipentaose derivatives from which the protecting groups were removed to provide di- to penta-saccharide analogs of the elusive Polysaccharide II. Because of the limitations of the monosaccharide donor-based chain extension we also investigated several donor/acceptor derivatives of the disaccharide kojibiose. The rationale behind this approach is that if successful, the chain is increased by two residues after each glycosidation. Reaction of these donors with partially protected glucosides having their HO-2 free afforded kojitriose derivatives in high stereoselectivity. On the other hand, attempted coupling of the kojibiose donors with kojibiose or kojitriose derivatives having a free HO-2 at their non-reducing end termini failed. Major products in these reactions included cyclo-kojibiose and trehalose-type tetrasaccharides.

Major Findings:

(a) We demonstrated that the prevalent monosaccharide components in polysaccharide fractions from Mycobacteria are arabinose, galactose and mannose. We identified a polysaccharide fraction which contains an mannose and glucose in approximately equal amounts together with a smaller proportion of arabinose. We also demonstrated the presence of a glycogen-like polysaccharide as one of the extracellular polysaccharides of Mycobacteria.

(b) We developed a method for the chemical synthesis of koji-oligosaccharides. We showed that the stepwise synthesis is the method of choice for the synthesis of such compounds. The failure to use [2+n (n>1)] block-synthetic approaches indicate extensive clashes between the acceptor and the donor. The immunochemical properties of the synthetic kojipentaose have been studied as described in Dr. John B. Robbins' report (Z01 HD 01311-02).

Proposed Course:

(a) The structure of the glucose-enriched fraction of M. tuberculosis will be studied using specific degradation instrumental analytical methods.

(b) Higher-order oligosaccharides corresponding to Polysaccharide II will be synthesized in a form that allows their covalent attachment to macromolecules. The conformation of the synthetic koji-oligosaccharides will be studied. These studies may reveal a conformational determinant of Polysaccharide II and may provide a better understanding of the molecular basis of the unique properties of this polysaccharide.

Publications:

Pozsgay V, Robbins JB. Synthesis of a pentasaccharide fragment of polysaccharide II of Mycobacterium tuberculosis. Carbohydr Res 1995; 277:51-66.

Dubois EP, Robbins JB, Pozsgay V. Chemical approaches to bacterial vaccines. Synthesis of mycobacterial oligosaccharide-protein conjugates for use as serodiagnostics and immunogens. Bioorg Med Chem Lett 1996; 7:1387-92.

Dubois EP, Neszmelyi A, Lotter H, Pozsgay V. A serendipitous synthesis of cyclokojibiose. Tetrahedron Lett 1996; 37:3627-30.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01315-02 LDMI

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Modulation of Protein and Cell Functions by Heparin/Heparan Sulfate

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A.L. Stone	Research Chemist	LDMI/NICHD
	E. Orvisky	Visiting Fellow	LDMI/NICHD

COOPERATING UNITS *(if any)*

Harvard Medical School, Boston, MA (R.P. Junghans); BEIP, NCRR (M.S. Lewis); LDDRD, NCI (J.B. McMahon); CPL, CC (M. Horne)

LAB/BRANCH Laboratory of Developmental and Molecular Immunity

SECTION Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS: 2.05	PROFESSIONAL: 1.5	OTHER: 0.55

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project elucidates the fundamental molecular architecture of heparins/heparan sulfates and studies the molecular basis of their specific modulation of proteins and cell membranes in diverse normal and diseased physiological systems, using various physical, biochemical, and biological approaches. We examined heparins as a mechanism of viral infectivity, studied structured on structure-function relations of a heparin-mimetic pharmaceutical composed of a mixture of sulfated oligoxylans (S-oligoS), which as heparin, inhibits the infectivity of HIV in vitro: 1) We established that the capacity to inhibit cytotoxity and sync-tium-forming infectivity of HIV-1 was governed by a structural specificity (HD 01315-01). Now, we find that the two antiviral capacities also expressed differential structural requirements (e.g., mass≥ ~5400 and ~ >4500, respectively). 2) We established that the minimal-sized potent antiviral component, CpF, was separable from S-oligoS with anticoagulation activity against thrombin. And that the S-oligoS also exhibit structural specificity in various anticoagulation reactions. 3) We showed S-oligoS contained multiple a D-GIcA moieties, that the Proton NMR analysis confirms this. The data indicate a new model structure, a tetrasaccharide repeat of three b 1-4 linked sulfated xylose units containing an a 1-2 linked 40Me sulfated D-GIcA, and in a fraction of S-oligoS yield a viral-inhibitory material (~900mg) without anticoagulant activity. [Total expected yield = ~900 mg.] Endotoxin assays showed no significant contamination. We are now able to develop a medium pressure LC system with good manufacturing practices for a large scale preparation of CpF or S-oligoS with specificity for other heparin-mimetic functions.

PHS 6040 (Rev. 5/92)

Project Description:

This project studies the fundamental molecular architecture of heparins and heparan sulfates (glycosamino polysaccharides of cells and the extracellular matrix) to elucidate the structural basis of their modulation of proteins and cell membranes in diverse normal and diseased physiolgical systems, using physical, biochemical and biological approaches (Z01 MH 013605-12-LNB). There is a current imperative in the field of the glycobiology of heparin to generate such knowledge and to conjoin it in a rational strategy for development of heparin-mimetic drugs against: tumor growth and angiogenesis, tissue inflamation, lymphocyte malfunction, viral infectivity, autoimmune disease, and the coagulation and complement cascades. Except for Rosenberg's elucidation of the structural specificity of heparin/heparin sulfate (H/HS) in anticoagulation, the structure-function relations of H/HS remain undetermined. We provided a model of structural elements in heparins which may apply to their diverse specificties and to those of the heparin-mimetic oligosaccharides as well. Current research focuses on the broadly heparin-mimetic sulfated xylans, which, as does heparin, inhibit the immunodeficiency virus, HIV-1, and many other functions of heparin as well. Structure-function relations of this oligosaccharide mixture are studied in two antiviral and three anticoagulant systems *in vitro*. Structual characterizations are by spectroscopic, ultracentrifugal, chromatograph-ic, and chemical analyses. Other, collaborative, studies included structural aspects of the soluble T-activation chain of ILR2 and of myelin basic protein (completed).

Objectives:

1. To study the structural basis for the heparin-mimetic capacities of the sulfated oligoxylans (S-oligoS), and to elucidate the specificities in their structure-function relations, particularly in their anti-HIV-1 capacities:

   a) AL Stone with JB McMahon, FCRDC and MS Lewis, NCRR

Procedures for purifying and characterizing many oligosaccharide components of the antithrombotic pharmaceutical SP54 (which result from degradation of the polysacchar-ide during the sulfation process) were established as described (Z01 MH 02593 02-3 LBG): LP permeation chromatography, quantitative spectroscopy (dye-coupling, titration) soluble formazan asay for HIV-1 cytotoxicity (50% effective anti-CT concentration (EC50)) and a syncytium-forming assay to quantitate the fusion reactions between infected and uninfected CD4 T-cells (anti-S-F EC50). These studies were followed by measurements of mass (ultracentrifugal analyses), glucuronic acid content (chemical reaction with o-nitro phenylhydrazide or carbazole), and anticoagulation activity (purified enzyme-anti-factor Xa and anti-thrombin assays and the clinical APTT test) (Z01 HD 01315-01). We first showed that the antiviral capacity of the S-oligoS was governed by a structural specificity, over a broad range of ~30000 to ≤2000 in mass. (This finding was confirmed subsequently by collaborators in another laboratory). Three types of anti-HIV-1 structures were identified and purified: 1) component CpFC, ~an eicosaccharide, highly active against the cytotoxic infectivity (CT) and the syncytium-forming infectivity (S-F) [EC50 = 50-100 ng/ml]; 2) CpF, the minimum-sized highly active S-oligoS (~ tetradeca-to-hexadecaS) EC50s = 250 and 50 ng/ml, resp., which had insignificant anti-coagulant activity vs thrombin; and 3) CpC, ~ a nonoS relatively highly active vs CT but not S-F, and anticoagulantly inactive.

The heparin-mimetic anti-CT and anti-S-F capacities among the S-oligoS appeared also to exhibit differential structural requirements. Analysis this year cofirmed this and showed that the highly active anti-CT capacity fell off at mass ≤~5400, while the anti-S-F potency first began to fall off at ~≤4500. There appears to be also a low mass component of relatively high anti-S-F activity, which is smaller than the low mass anti-CT-active component.

The chromatographic purifications were highly reproducible and we now compare the results of the various assays by normalization to the "elution volume fraction" (elution volume - void volume/total elution volume - void volume) of the various samples.

A manuscript, "Structure-function relations of sulfated xylan oligosaccharides in the inhibitionof human immunodeficiency virus - 1 infectivity *in vitro*" by AL Stone DJ Melton and MS Lewis is in revision (with enlargement) for Glycoconjugate Journal; Preparation of manuscript "Differential inhibitory capacities of oligosaccharides comprising sulfated xylan in the inhibition of HIV-1 cytotoxicity and syncytium-forming infectivity *in vitro*" by AL Stone DJ Melton, Krebbs B, and J McMahon continues.

b) AL Stone

A previous study led us to propose that the heparin-mimetic capacity of the components might redound from glucuronic acid-containing structures and, contrary to the general expectation from the ratio of Xyl to GlcA of ten in the original xylan, we subsequently showed (by the chemical reactions) that most of the S-oligoS contained multiple GlcA moieties. This year we have confirmed this higher content of GlcA by setting up a proton NMR analysis. These show that there is an average of about 3 Xyl to 1 GlcA in several components, including CpF, and give promise that this method will provide additional new information about the structure-function relations of heparin-mimetic oligo-S. Taking the GlcA data together, we now think that a repeat of a tetrasaccharide of three Xyl containing a GlcA substituent is a structural requirement for the mimicry of heparin by the sulfated xylan.

c) AL Stone with E Orvisky

To devise an upscaled method to prepare the minimally-sized, highly antivirally active CpF in quantities sufficient to yield an agent potentially suitable for testing against the AIDS virus, using the biological, chemical and physical characterisctics above and in Z01 01315-01 LDMI; to develop forefront additional methods for characterization and identification of S-oligoS:

Last year we showed that direct upscaling using LP chromatography could provide a means of obtaining larger batches of CpF. This year we completed rechromatography and analytical characteristization of four segments of this preparation and obtained more highly purified CpF from the two center segments in good yield. Three series of upscaled preparations of CpF (two started last year) were variously modified to provide for possible clinical application. Modification of gels by autoclaving or ethanol pretreatment to minimize pyrogenic contamination variably altered their chromatography, especially ethanol. A reproducible CpF product is being harvested, however, by use of the estab-lished values of characteristic "markers" for CpF (above) and newly developed markers (time was spent on establishing additional methods for the study of S-oligoS and charac, terization of CpF, see below). Each series of preparation consists of 5 to 7 runs, each of which separately analyzed. Now appropriate fractions are being combined to yield the desired component from each series. Either BioRad P10 or P6 LP upscaling provided a purification of CpF. After satisfactory analyses of the third series (ethanol pretreatment) the CpF will be combined and assessed for *in vivo* studies and possible clinical application. Endotoxin assays of the various preparations and gel systems indicate that with careful prevention of contamination, pretreatment of gels can be avoided to allow a more reproducible system.

While the above upscaling provides sufficient component for further study, it would not be a feasible means of obtaining large amounts for clinical use. We are exploring the potential usefulness of a medium pressure LC system for preparing larger batches of CpF. This, however, involves different gels with different physical characteristics of separation.

Dr. Eduard Orvisky using our data on previoulsy unavailable highly sulfated oligoS mass standards has developed a HPLC method of analysis of the S-oligoS by their relative retention times. In this system, the range of retention times for our anayses is ~7.5 to 9.5 minutes and the individual retention times are generally exactly reproduced three times. This has provided another sensitive characteristic for identifying CpF and other components of interest in our system. Additionally, he has produced the first calibration curve for the determination of the mass of any such sulfated oligosaccharide from the retention time. Dr. Orvisky is also developing a PAGE system for analysis of S-oligoS.

We intend to confirm our ultracentrifugal data on mass by mass spectrometry on appropriate samples. Dr. Orvisky will extend our previous studies on CD4 cells, which approached the questions concerning putative of S-oligoS receptor(s) and the role such might play in inhibition of HIV-1 by S-oligoS.

2. AL Stone with MacD Horne

To elucidate the structure-function relations of the anticoagulant capacities of the mixture of S-oligoS, the anticoagulant activity of the various S-oligoS fractions and components were measured by established *in vitro* assays of the inhibition of antithrombin-dependent inhibition of thrombin and Factor Xa, and by the clinical APTT coagulation test as well.

The anticoagulant capacities of the S-oligoS in this antithrombotic pharmaceutical also were seen to exhibit differential potencies. Moreover, the dependency on mass of the anti-thrombin capacity differed from that of the anti-Factor Xa, which could be expected from the known structure-function relations of heparin. Anti-thromin activities of 26 - 73 heparin units/mg, based on a heparin standard of 154 units/mg, were exhibited by the highest molecular weight components (>~7000) [low molecuar weight (LMW) heparin, Lovenox exhibits 23 HU/mg]. This capacity declined sharply at elution volume fraction >0.250 and thus was sharply separated from CpF which has an elution volume fraction = >0.32-0.36 for the decline of anti-CT potency and >0.39 for the decline of the anti-S-F potency). The anti-factor Xa potencies of the S-oligoS were all relatively low, varying from ~0.4 to 0.9 heparin units/mg (about 1% that of LMW heparin which had a value of 74) in the range of elution volume fractions ≤, ~0.56 above which the anti-Xa activity declines to an insignificant value. A low level of antithrombotic activity, therefore, appears to reside non-specifically over a range of components in this mixture.

Fractions from upscaled preparations are now subjected to an automated test that is used clinically to evaluate coagulation capacity of a patient's plasma. The increase over normal in seconds of coagulation time/ug sample under the conditions of the test is the APTT slope. In heparin therapy the accepted slope would be ~11.5 (a level of two times the normal time of 25-30 sec). The highest MWt S-oligoS exhibit a slope of ~6. Fractions in the CpF range show slopes of about 4 and the value declines gradually in the range correlating with elution volume fraction > 0.5. Others have shown that the elevation of APTT time by SP54 is rapidly reversible in humans. If the APTT slope of purified CpF were less than 4, a level of at least 5 ug/ml in the blood would not increase the APTT time more than 1.5 fold, a level that would be tolerable for use in children by some NIH clinicians.

The methods and findings above provide a basis for: 1) study of molecular mechanisms involving S-oligoS in the effects of HIV-1 that kill immune cells; 2) continued development of a potential, inexpense adjunt to AIDS therapy (CpF); and 3) improvement of clinical usefulness of SP54 in anticoagulation and in tumor therapy by isolation of more specific S-oligoS components, as well as for elucidating the structure-function relations of H/HS and S-oligoS. This project will continue.

3. AL Stone, RP Junghans collaboration with MS Lewis.

To determine the molecular form of the soluble T-activation atigen (Tac) chain of the interleukin 2 receptor (IL2R), a purified recombinant Tac was examined by size exclusion chromatography, velocity-sedimentation and sedimentation-equilibrium ultracentrifugation analysis and low ultraviolet circular dichroism spectroscopy as described last year (Z01 HD 01315-01 LDMI). Findings resolve controversy about possible oligomerization of Tac and point out that the biologically relevant form is a momomeric protein. Knowledge of the molecular form os soluble Tac is required to understand the regulation and process of lymphocyte activation in vivo. A manuscript was published this year.

4. AL Stone, GE Deibler collaboration, LCM, NIMH.

Myelin basic protein (MBP) is the encephalitogenic antigen in the experimental autoimmune model for study of multiple schlerosis. Brain myelin contains several isoforms, in particular a phosphorylated form. Results showed that in vivo phosphorylation to PT96 produced an increase in stable folding of 18.5 kDa hMBP up to ~30%, which was likely transition to a class B type II beta turn and beta-structure. These findings supported our suggestion that conformational adaptability associated with isoforms of hMBP may subserve a structural integrity of myelin. Manuscript was in press last year.

Significance to Biomedical Research and the Program of the Institute:

Several findings revealed in these studies bear significantly on Biomedical Research particularly, but not only, in the area of antiviral agents and mechanisms:

1. The heparin-mimetic SP54 (a sulfated xylan, antithrombotic pharmaceutical) has been shown to inhibit various viruses (including HIV-1 and the Scrapie virus) and mammalian tumors *in vitro*. Realization of this potential in clinical application, however, has been limited. We now show that this mixture contains S-oligoS which have structural specificty in various heparin-mimetic functions, and which are probably separable, as are CpF, from the antithrombin-active S-oligoS. As in research on heparins, use of such heparin-mimetic

mixtures must now be considered inconclusive and to be avoided. This finding will likely stimulate new thinking and approaches in the clinical applications of sulfated xylans.

2. We now suggest a molecular basis for the heparin-mimetic capacities of sulfated xylan, which newly offers an approach to more feasible semi-synthetic production of various S-oligoS having particular functional specificiies.

This project works towards obtaining a clinically useful, inexpensive adjunt to AIDS therapy in adults and children, while elucidating basic molecular mechanisms of heparin-like actions.

Publications:

Deibler GE, Burlin TV, Stone AL. Three isoforms of human myelin basic protein: Purification and structure. J Neurosci Res 1995; 41:819-27.

Junghans RP, Stone AL, Lewis MS. Biophysical characterization of a recombinant soluble interleukin 2 receptor (Tac). J Biol Chem 1996; 271:10453-60.

LDN

NICHD Annual Report
October 1, 1995 to September 30, 1996

Laboratory of Developmental Neurobiology

The space renovations involved in housing the programs of Drs. Fields and Lu, initiated a year ago or more ago are only now being completed. Difficulty with timely renovations are a troubling aspect of NIH function, but the now-complete labs will be excellent facilities. Dr. Denes Agoston has moved to temporary space in Bldg. 36, where his group continues to be very active and will be supported by NICHD until June of 1997.

Dr. Douglas Brenneman's Section on Developmental and Molecular Pharmacology continues its study of neurotrophic materials with potential clinical applications. Cloning of the activity dependent neurotrophic factor (ADNF) related proteins has been achieved and trophically active peptide fragments of these proteins identified.

The Section on Cellular Neurobiology headed by Dr. Y. Peng Loh is focussing on the problem of sorting of prohormones into the regulated secretory pathway. They have identified the sorting signal in the prohormone sequence as 13 amino acid amphipathic loop. They have now characterized the sorting receptor in Golgi and secretory granule membranes as a 54 KD protein and have purified and cloned this molecule.

Dr. Phillip Nelson and colleagues in the Section on Neurobiology emphasize mechanisms involved in activity dependent synapse reduction. They have shown that the crucial role played by thrombin in this process is mediated by the thrombin receptor and involves the action of a protein kinase. Activity independent cellular and molecular elements related to map formation in the visual system are also under investigation.

Dr. David Klein and co-workers in the Section on Neuroendocrinology have succeeded in cloning the gene for N-acetyltransferase, the rate limiting enzyme in the biosynthetic pathway for melatonin. They are rapidly exploiting this breakthrough result in studies of transcriptional regulation of the genes expression and analyses of the organization of the gene as well as its phylogenetic variations.

Dr. Elaine Neale's Section on Cell Biology continues to pursue the process of neurotransmitter release from synaptic terminals by using the clostridial neurotoxins. These toxins selectively cleave specific proteins involved in exocytosis. Botulinus toxin C also exhibits a potent neuronal killing effect which may be related to its proteolysis of syntaxin and consequent effect on syntaxin-related calcium channels.

Dr. Andres Buonanno's Unit on Molecular Neurobiology has identified regulatory regions in the genes for contractile proteins of slow and fast skeletal muscle which are critical in determining the responses of these genes to different patterns of activity in the muscles. Switching of glutamate receptor subunit expression during development is being studied in preparations of cerebellar granule cells.

Dr. Denes v. Agoston and colleagues in the Unit on Molecular Control of Neurodifferentiation continue to focus on regulation of the enkephalin gene as a model for neuronal commitment to a given neurotransmitter phenotype. Their experiments have shown a number of protein-DNA interactions related to the progressive restriction of the enkephalin phenotype. Three brain and development specific nuclear proteins are being examined in detail.

The Unit on Neurocytology and Physiology under the direction of Dr. R. Douglas Fields is investigating how specific patterns of electrical activity in neurons modulate the expression of genes important for nervous system function during development. The expression of a cell adhesion molecule, L1, is one such activity-modulated gene. The behavior of the cell biologic cascades responsible for the coupling of electrical activity to gene expression are also under investigation.

The Unit on Synapse Development and Plasticity, led by Dr. Bai Lu, is studying the mechanisms involved in the formation of specific synapses in the nervous system. The influence of trophic factors such as Brain Derived Neurotrophic Factor on synaptic function and structure is of interest and the expression and activity of other molecules important for synaptic plasticity is under investigation.

Section on Developmental and Molecular Pharmacology

Trophic Factors

The Section on Developmental and Molecular Pharmacology, led by Douglas Brenneman, continues to focus on the neurotrophic and growth-regulating actions of vasoactive intestinal peptide (VIPP. The perspective emerging from this group is that VIP is an integrative peptide influencing many developmental processes through its function as a secretagogue for many ontogenic regulators. Of particular significance is their work on a new class of growth factors released from astrocytes: activity dependent growth factors. These substances are apparently structurally related to stress proteins. In addition, this group continues to exploit the use of whole embryo cultures to define and explore the growth-promoting mechanisms elicited by VIP and related proteins.

Activity Dependent Neurotrophic Factor

Previous studies by this group have indicated that a survival-promoting protein acting at femtomolar concentrations was released from astrocytes by low concentrations of VIP. This protein was biochemically purified and was named activity dependent neurotrophic factor (ADNF) because the test cultures, in which the factor was detected, had to be electrically blocked in order to observe the survival-promoting activity. During the past year, it has become apparent that several proteins released from glia have this property. In collaboration with Illana Gozes and Meirav Basan of Tel Aviv University, a clone of an ADNF-like molecule was found. Expression cloning of a P19 library screened with two antisera to ADNF resulted in a positive clone with a unique nucleotide sequence that contained 2358 base pairs with an open reading frame of 811 amino acids. The fusion protein from this clone increased neuronal survival in cerebral cortical cultures treated with tetrodotoxin. The protein encoded by this clone was named ADNF-3, because of its similar biological activity and homologous structure to ADNF-1 related peptides. Like the biochemically isolated ADNF-1, ADNF-3 prevented neuronal cell death associated with beta amyloid peptide, a toxic substance implicated in the etiology of Alzheimer's disease. Interestingly, a bimodal dose response was observed for this protective effect, suggesting a neuroprotective mechanism involving several biochemical pathways.

One of the remarkable findings in this group's investigation of ADNF I was the identification of an active peptide that retained the potent neuroprotective properties exhibited by the whole ADNF protein. The drug delivery advantages of having a peptide versus a protein are critical to the development of these compounds as therapeutic agents. An active peptide of ADNF-3 was identified, which was homologous in sequence to the neurotrophic peptide known for ADNF-1. Gretchen Gibney also discovered an active ADNF-like peptide during the course of her work in cloning ADNF-like proteins from human cDNA libraries. This emerging picture of highly potent, neurotrophic peptides should form the basis for drug development in the treatment of neurodegenerative disease. This is particularly significant in that neuronal cell death associated with two clinically relevant neurotoxic substances (beta amyloid peptide and the envelope protein from the human immunodeficiency virus) can be prevented by co-treatment of test cultures with the neurotrophic peptides. In addition, with the discovery of the ADNF-3 clone, mapping of this substance in embryonic tissue and the brain has now become feasible.

Early Pregnancy Factor and Embryonic Growth

The pioneering work on the growth-regulating action of VIP was conducted by this group utilizing whole embryo cultures. With this technique, compounds suspected of having regulatory roles during early post implantation embryos can be screened and studied. Previous work by Joanna Hill and Gordon Glazer indicated that ADNF, like VIP, had a marked effect on embryonic growth during E8-10 in gestation. Growth was measured by cross sectional area, somite number, DNA and protein content, and gene expression of growth factors and cyclins.

ADNF has sequence homology with one of the stress proteins, heat shock protein 60 suggesting that proteins belonging to the stress protein family can be found in the extracellular compartment. Continuing in this line of research, this group has also begun studies on early pregnancy factor, a substance in maternal serum during pregnancy which is identical to chaparonin-10 (cpn10), and is also found extracellularly. In collaboration with Alice Cavanagh, cpn10 was shown to produce growth stimulation in studies conducted by Susan Lee and Joanna Hill. The magnitude of the growth stimulation was similar to that observed with VIP or ADNF. An equally important observation made by this group suggested that endogenous cpn10 was necessary for embryonic growth. Treatment of E9.5 mouse embryos with neutralizing antiserum to cpn10 produced a concentration-dependent decrease in embryonic growth. This finding indicates that a cpn10-like substance is present in the embryo culture and that the substance is obligatory for embryonic growth at this stage of development. Furthermore, in situ hybridization studies demonstrated that mRNA for cpn10 was evident in extraembryonic membranes and in the floor plate of the neural tube at mid-gestation. This is highly significant in that the floor plate is critical to the regulation of organogenesis. In accompanying studies, the floor plate was also found to be highly enriched in VIP binding sites, further supporting a regulatory role for VIP at this stage of development.

Section on Cellular Neurobiology

The Section on Cellular Neurobiology led by Dr. Y. Peng Loh studies the intracellular trafficking, enzymology and regulation of biosynthesis of neuropeptides, with specific focus on the ACTH/endorphin family of peptides. These peptides play a role in intercellular communication and neural development. The ACTH/endorphin family of peptides are cleaved from a common prohormone, pro-opiomelanocortin (POMC). POMC is synthesized at the rough endoplasmic reticulum, transported to the Golgi apparatus and sorted at the trans-Golgi network (TGN) into secretory granules of the regulated secretory pathway where it undergoes proteolytic processing and subsequent regulated secretion of the products. A highly specific set of proteases are involved in cleaving this prohormone, in a tissue-specific manner, to yield different peptide products in brain, intermediate lobe and anterior lobe of the pituitary.

Mechanism of Sorting Prohormones to the Regulated Secretory Pathway

The Section continues to investigate the molecular signals and mechanism(s) involved in the sorting of prohormones and pro-neuropeptides to the secretory granules of the regulated secretory pathway. A sorting signal motif consisting of a 13 amino acid (N-POMC$_{8-20}$) amphipathic loop stabilized by one disulfide bridge (Cys$_8$-Cys$_{20}$), that is both sufficient and necessary for targeting POMC to the regulated secretory pathway has been identified. Molecular modeling and NMR studies done in collaboration with Dr. C. Snell (Sandoz Inst., London) and Dr. A. Bird (NCI, Frederick) respectively, show that the loop contains four highly conserved residues, Asp_{10}, Leu_{11}, Glu_{14}, and Leu_{18} which are exposed and may be involved in ionic and hydrophobic interactions with a sorting receptor. Substitution of these 4 residues with Ala_{10}, Ser_{11}, Ala_{14} and Ser_{18} eliminated the targeting of this POMC mutant to the regulated secretory pathway indicating the physiological importance of these four residues in sorting POMC at the TGN. A similar sorting signal motif containing an amphipathic loop within residues 6-24 of pro-enkephalin, stabilized by one disulfide bridge (Cys_6-Cys_{24}), has also been identified and shown experimentally to be functional in targeting this prohormone to the regulated secretory pathway. A sorting receptor has recently been characterized in Golgi and secretory granule membranes and identified as a ~54 kD protein. The N-POMC sorting signal motif bound specifically to this receptor at an optimum pH of 5.5-6.5, consistent with the acidic pH of the TGN where sorting occurs. Other regulated secretory pathway proteins such as pro-insulin, pro-enkephalin and chromogranin A, but not constitutively secreted proteins also bound to this receptor with similar kinetics to the POMC sorting signal. Thus, this appears to be a common sorting receptor for many prohormones. The receptor has now been purified and cloned. Antisense knock out of expression of this receptor in Neuro2a cells resulted in the lack of targeting of transfected POMC to the regulated secretory pathway. Thus, our studies have identified consensus sorting signals on prohormones and a physiologically functional sorting receptor for these signals, providing overwhelming evidence in support of a receptor-mediated mechanism for sorting proteins to the regulated secretory pathway.

Characterization of Novel Pro-hormone Converting Aspartic Proteases

Our group has continued to study the proteolytic enzymes involved in prohormone conversion to active hormones with specific focus on a novel class of prohormone converting aspartic proteases. These enzymes have unique specificity for mono- and paired basic residues of prohormones and they include yeast aspartic protease 3 (YAP3p) and mammalian pro-opiomelanocortin converting enzyme (PCE). We showed that YAP3p and PCE are structurally related based on immunological cross-reactivity of antiserum against YAP3p with PCE. They are also structurally related to pepsin and hence these enzymes have recently been named YAPsins. We have studied YAP3p (YAPsin 1) extensively as a model enzyme of the YAPsin family. Biosynthesis studies of YAP3p showed that it is synthesized as a pro-enzyme and is autocatalytically cleaved to yield a more highly active enzyme. A further cleavage takes place to yield two subunits which are linked by a disulfide bridge to form a heterodimer. The specificity and catalytic efficiency (Kcat/Km) of purified YAP3p for 15 different substrates were compared. YAP3p cleaved at paired basic residues more efficiently than a substrate with a monobasic residue at the cleavage site and another basic residue at P6. The efficiency was greatly enhanced by additional upstream and downstream basic residues surrounding the cleavage site. In particular, a basic residue in the P2' position enhanced the efficiency greatly. Molecular modeling of YAP3p done in collaboration with T. Blundell's group (Cambridge University) showed an acidic Asp residue in the S2' pocket which could strongly attract the basic residue in the P2' site of the substrate. The model was supported by substitution studies showing that substrates with an Asp in the P2' were cleaved very inefficiently since the two Asp residues would repel each other. This first structure/activity study of a member of the YAPsin family of prohormone converting enzymes has provided a better understanding of the type of natural substrates this class of enzymes can most efficiently cleave *in vivo* and hence their function.

Two immunologically-related mammalian homologues of YAP3p (mol. wt. 70 kD and 90 kD) have been identified in brain and pituitary. Immunocytochemistry in combination with *in situ* hybridization have revealed co-localization of YAP3p immunoreactivity with CCK mRNA in hippocampal and cortex neurons and vasopressin mRNA in supraoptic neurons, indicating a role of YAP3-like enzymes in prohormone processing in the CNS. In pituitary, YAP3p immunoreactivity was found restricted to corticotrophs, unlike the subtilisin-like prohormone convertases, indicating a role of YAPsin enzyme(s) in POMC processing. Recently, we have attempted to clone mammalian YAPsins, using the antibody against YAP3p to screen a mouse brain expression library. Two clones have been obtained and are currently being sequenced.

Section on Neurobiology

Development of Synaptic Circuits

The epigenetic role of nervous system activation in determining important aspects of brain development remains a primary focus of Dr. Phillip Nelson's Section. The structural and functional properties of the nervous system are powerfully influenced by environmental inputs during early 'sensitive' periods and understanding the mechanisms involved in the environmental shaping is an important goal of the Section. In particular, the massive and widespread reduction in an initially redundant oversupply of the synapses linking neurons in functional circuits is essential for efficient nervous system operation. Arguably, the major portion of the experiential "tuning" of synaptic circuits is due to this synaptic "pruning".

The mammalian neuromuscular junction is the preparation where this process of activity dependent synapse reduction (ADSR) has been most intensively studied and we have developed a tissue culture system involving two independent populations of cholinergic motoneurons which innervate a common population of muscle fibers. Different patterns of neural activation can be imposed electrically for periods of several days and the consequences of activation on synapse strength evaluated. Pharmacologic interventions are also readily achieved.

We have shown that a substantial (~%50) reduction in the number of functional synapses can be produced by 24 hours or more of patterned electrical stimulation in our system and have taken this robust phenomenon as our assay for probing the mechanism responsible for the ADSR. We have previously shown that the ADSR requires the activity of a serine protease and identified this protease as thrombin, since the process of ADSR is completely blocked by the specific thrombin inhibitor, hirudin, at nanomolar or lower concentrations. The mechanism of action of thrombin is mediated by at least two quite distinct processes. As a critical step in the blood clotting cascade, thrombin catalyzes the conversion of fibrinogen to fibrin, and in principle could function as a serine protease cleaving any of a number of substrate proteins. In addition, thrombin produces several of its effects via a proteolytic interaction with the thrombin receptor, a membrane protein. Cleavage of the N terminal, extracellular portion of this molecule by thrombin produces a new, exposed terminal sequence which acts as a "tethered ligand" to activate the receptor. In some systems the thrombin receptor activation produces activation of protein kinase C and perhaps other kinases. The active terminal sequence of the receptor has been determined and the corresponding peptide is available and serves as an agonist for the receptor. In addition, a peptide identical in amino acid composition to the active one but with two of the residues reversed is available; this is completely inactive in other test systems.

We have utilized these reagents to test whether the action of thrombin in mediating the ADSR is via the thrombin receptor or by some other proteolytic action. We find that synapse reduction in our system is produced by the active Thrombin Receptor Activating Peptide (or TRAP) in a dose and time dependent fashion with 10-6 M SFLL (our designation for the active peptide) giving maximal synapse loss and significant loss occurring in 24 hours. The inactive control peptide (designated FSLL) produces no loss. Indeed, it appears that there may be some protective effect of FSLL.

In analogy with other systems, if thrombin effects are mediated by the thrombin receptor, then protein kinase C (PKC) or other kinases might be expected to be involved. To test for this possibility, we have used three kinase inhibitors, staurosporine, H-7 and calphostin. Both staurosporine and H-7 prevented the synapse loss produced by SFLL, while calphostin at up to 500 nanomolar concentration did not. Importantly, staurosporine at 3 nanomolar concentration, substantially blocked the synapse reduction produced by electrical activation. Thus both the naturally evoked and the TRAP-elicited ADSR appear to involve PKC activation. More specific identification of the kinase species awaits further experimentation.

Activity independent cues play a critical role in the development of synaptic circuits and we have utilized the retinotectal and retinocollicular systems of the chick, mouse and rat in searching for these cues. Three categories of growth cone responses have been identified by time lapse photography in these systems. These have led us to 3 categories of guidance molecules which are differentially distributed amongst tectal (chick) and superior colliculus (mouse). Neurons in both phyla have repellant cues localized to one particular target region, whereas glia from chick and mouse appear to express qualitatively different classes of guidance components.

Section on Neuroendocrinology

The Function of the Pineal Gland

This program has been dedicated to understanding how the production of melatonin is regulated. This is important for two reasons. One is that basic knowledge about how the synthesis of this hormone is regulated is required to understand the role melatonin plays in physiological processes. The second is that the production of this hormone is regulated by a highly complex neural system which enables remarkably rapid and precise regulation. Because of this, knowledge gained from these studies may be applied to other neural systems.

The Section on Neuroendocrinology directed by Dr. David Klein has made a major advance by cloning a melatonin synthesizing enzyme that regulates large changes in the rate of melatonin production, serotonin N-acetyltransferase. The large and rapid changes in the activity of this enzyme control similar change in melatonin production and the levels of melatonin in the blood. Melatonin levels and serotonin N-acetyltransferase activity are high at night and low during the day, providing the body with a hormonal analog of darkness at night. This is used to entrain physiological rhythms with photic changes in the environment, typically linked to seasonal changes in day length.

Analysis of the genetic code of the enzyme indicates that it represents a new subfamily within a larger weakly related family of acetyltransferases. No closely related acetyltransferases, however, have been identified. Analysis of the deduced amino acid of the protein in the chicken, human, rat, sheep, monkey, and trout has revealed the enzyme is ~23.5 kDa and is characterized by several highly conserved regions. These have been putatively identified as: a catalytic region, which is rich in histidines; an arylalkylamine binding region, which has the most highly conserved sequences; and, an acetyl CoA binding region, which shares homology with other acetyltransferase molecules. In addition, there are five highly conserved thiols and N- and C-terminal phosphorylation sites.

Studies on the rat, sheep and chicken pineal gland revealed that activity is in all cases influenced by cyclic AMP acting through post transcriptional mechanisms. Large decreases in activity can occur without associated decreases in mRNA. In all cases, large changes in activity are associated with similar changes in total enzyme protein. Although these features of regulation are conserved, the role of transcriptional mechanisms - i.e. changes in mRNA levels - varies on a species-to-species basis. In the rat, serotonin N-acetyltransferase mRNA is not detectable during the day. The increase in enzyme activity and protein requires a 150-fold increase in mRNA. In sheep, the situation is markedly different: serotonin N-acetyltransferase mRNA is always present at high levels. The chicken is intermediate, in that the rhythm in mRNA is about 10-fold.

Studies are now aimed at learning more about the structure and function of this enzyme, and the mechanisms involved in regulating the disappearance of enzyme protein. Related to this are efforts aimed at identifying human polymorphisms that might alter the function of this important enzyme, which would provide an approach to the question of the physiological role of melatonin in humans. Other efforts are aimed at understanding the molecular basis of the system that regulates expression of the serotonin N-acetyltransferase gene in the chicken, because there is a clock in the pineal cell linked to the rhythmic expression of this gene. Learning more about this mechanism might provide an avenue towards the important goal of learning the molecular basis of the master oscillator in man's brain located in the suprachiasmatic nucleus, The Mind's Clock.

Section on Cell Biology

Probes for Transmitter Release

The Section on Cell Biology under the direction of Dr. Elaine Neale has continued to study the similarities and differences among the clostridial neurotoxins. These are toxins which cause clinical tetanus and botulinum poisoning by blocking specific neuronal functions. Each of the toxins blocks the release of neurotransmitters as a consequence of the proteolysis of specific proteins required for the docking and fusion of synaptic vesicles with the presynaptic membrane. These toxins are of interest to neuroscientists and cell biologists because they can be used as tools to modulate synaptic activity and to study membrane movements within cells. Importantly, several of the botulinum toxins are used clinically to relieve the muscle spasms which characterize a variety of disorders.

Dr. Williamson has pursued her studies of the C serotype of botulinum neurotoxin. This serotype has not been studied extensively, perhaps because it is not associated with human botulism and is not utilized for clinical applications. In her efforts to characterize all of the serotypes, she noted evidence of neurotoxicity unique to botulinum neurotoxin C. With prolonged (up to five days) exposure, mature neurons exhibit severe degeneration, and neurons in newly plated cultures do not survive. Electron microscopy reveals that synaptic terminals show the first clear signs of degeneration. Dr. Williamson believes that the toxin's proteolysis of syntaxin may affect not only synaptic vesicle fusion, but also may alter the activation state of syntaxin-associated calcium channels allowing unregulated influx of calcium. Preliminary studies with ^{45}calcium support this hypothesis. Further studies aimed at understanding the basis of the toxin-induced nerve terminal deterioration undoubtedly will provide information about the role of syntaxin in events critical for neuron survival, and indicate whether botulinum neurotoxin C offers an advantage over the other serotypes for use in the therapy of muscle spasm disorders.

Continued neuronal function depends on the recycling of synaptic vesicle membrane in order to maintain a pool of releasable vesicles. Synaptic vesicle membrane that has fused with the presynaptic membrane in response to stimulation is thought to be retrieved by a process of endocytosis that is tightly coupled to the exocytotic event. All of the clostridial neurotoxins block vesicle fusion and consequently block synaptic vesicle membrane retrieval, with the singular exception of the A serotype of botulinum neurotoxin. Cultures exposed to botulinum neurotoxin A and examined biochemically, immunohistochemically, and microscopically, show evidence of a block in synaptic vesicle fusion and neurotransmitter release. However, optical and electron microscopic techniques which detect endocytosis indicate that potassium stimulation in the presence of calcium triggers synaptic vesicle membrane uptake into the nerve terminal. Recent quantitative studies using uptake of horseradish peroxidase to identify newly recycled vesicles. demonstrate a) the ability of botulinum neurotoxin A to block the exocytosis of synaptic vesicles; b) the capacity of toxin-blocked cultures to respond to stimulation by endocytosing synaptic vesicle membrane; and c) the dependence of both exocytosis and endocytosis on extracellular calcium. Thus, for synaptic vesicle recycling, it is not obligatory that endocytosis be preceded immediately by an exocytotic event. These findings lead to obvious questions about the source of membrane that is recycled into synaptic vesicles, and future experiments will address this issue.

Each of the clostridial neurotoxins is thought to bind to a specific membrane receptor as a first step in toxin entry into the nerve terminal. Polysialogangliosides on the neuronal surface membrane 'fix' tetanus toxin although it is not clear that the ganglioside/toxin interaction is required for toxin internalization. Ms. Aldaia, under the guidance of Dr. Williamson, initiated experiments using a drug to inhibit specifically the synthesis of gangliosides. That this drug offers significant protection of spinal cord neurons from the effects of tetanus toxin is taken as evidence that gangliosides play an important role in tetanus intoxication.

Dr. Williamson has begun to assess the relative potency of the clostridial neurotoxins at the nerve-muscle synapse; i.e., the site of action in vivo of the botulinum neurotoxins. Preliminary experiments suggest that the botulinum neurotoxins are relatively more potent than tetanus toxin in this system, in contrast to her findings in spinal cord cell cultures. Once toxin action at the nerve-muscle synapse is characterized thoroughly, this system will be used to study differences between botulinum and tetanus toxin uptake and intracellular routing.

In spinal cord cell cultures, as in vivo, tetanus toxin acts more efficiently to block the function of inhibitory neurons before that of excitatory neurons. In a collaborative study, Dr. Williamson has looked for a similar differential action of a specially constructed chimeric toxin. In the chimeric toxin, the membrane-binding domain of tetanus toxin is substituted with an anthrax toxin fragment capable of binding to an alternative site on both inhibitory and excitatory neurons. The chimeric toxin is equally effective in blocking the release of inhibitory and excitatory neurotransmitters. Thus, toxin receptors appear responsible not only for the preference of tetanus toxin for spinal cord synapses over the nerve-muscle synapse, but also for inhibitory spinal cord synapses over excitatory spinal cord synapses. The identity of the receptor(s) for any of the clostridial neurotoxins has yet to be identified.

The Section on Cell Biology plans to use nerve-muscle co-cultures for a number of experiments with the clostridial neurotoxins. However, the primary driving force for the optimization of this system is its requirement for studies of synapse stabilization / elimination as described by Dr. Phillip Nelson. Dr. Parfitt has introduced improvements in chamber design which allow enhanced neurite growth into the muscle compartment, which reduce the likelihood of fluid mixing across the compartments, and which provide a glass growth substrate for improved optical resolution. He has written software to drive a motorized microscope stage that will be used in several applications.

Dr. Williamson is involved in a collaboration to characterize the development of three isoforms of Na^+K^+-ATPase and of two subunits of the voltage-gated N-type calcium channel. These studies use specific antibodies in immunohistochemistry of spinal cord cell cultures at various times during development, and have provided new information about cell-type and developmental stage specific patterns of distribution. Finally, with the arrival of Dr. Qun Li, studies to examine the effect of neuronal activity on the development of neuronal structure are being pursued.

Unit on Molecular Neurobiology

Molecular Mechanisms Regulating Neural and Neuromuscular Plasticity

After neural circuits are established during synaptogenesis, the strength of connections made between neurons and their targets continue to change during development. Neural plasticity is essential for animals to adapt to changing environmental demands, and underlies processes such as learning and memory. Signals derived from presynaptic neurons in the form of electrical activity or neurotrophic factors selectively regulate plasticity by influencing the transcription of genes in postsynaptic cells. The goal of the Unit on Molecular Neurobiology, directed by Andres Buonanno, is to understand how transsynaptic signaling is coupled to the specific regulation of genes that ultimately strengthen or weaken the function of neural circuits. To this end, we have identified and analyzed the expression of genes that are differentially regulated by distinct patterns of electrical activity and neurotrophic factors. Two model systems have been exploited in these studies: we have analyzed how different depolarization frequencies selectively modify the contractile properties of slow- and fast-twitch skeletal muscles, and in the central nervous system, we have studied how neurotrophic factors and activity differentially regulate N-methyl-D-aspartate receptor (NMDA R) subunit genes. Cis-acting elements and transcription factors that mediate the selective responses to activity have been studied by analyzing DNA sequences in transgenic mice and transfected cells, and by using electrophoretic mobility shift assays. Common pathways coupling extrinsic signals to transcription in excitable cells are beginning to emerge from our studies.

Neuromuscular Plasticity

Motoneurons are matched to their appropriate types of muscle fibers during synaptogenesis to generate functional motor units. The contractile properties of skeletal muscle are regulated by motoneuron innervation but change in response to changing functional demands, during aging and in neuromuscular diseases. We have shown that electrical stimulation of denervated muscles with slow (10 Hz) and fast (100 Hz) frequencies, mimicking the firing patterns of motoneurons, is sufficient to govern the contractile properties of slow- and fast-twitch fibers by specifically regulating genes encoding different types of contractile proteins. The analysis of DNA sequences regulating the fiber-type-specific expression of the troponin I genes by Drs. Soledad Calvo, Manabu Nakayama, Jun Cheng and Jimmy Stauffer, has led to the identification of two enhancers that confer specific expression in either the slow- or fast-twitch muscles of transgenic mice. Using electrophoretic mobility shift assays, the nature of the transcription factors binding regulatory elements in the enhancers has been analyzed. These elements are being used to isolate the transcription factors underlying the changes in muscle phenotype in response to the patterned electrical signals elicited by the nerve.

Plasticity in the Developing Cerebellum

Activity-dependent signaling through NMDA receptors, primarily transduced by calcium, plays a critical role in the refinement and strengthening of neural connections during synaptogenesis. Signaling through these receptors controls the expression of down-stream genes that underlie neural plasticity. Since the electrophysiological properties and signaling through the NMDA R channel depend on its subunit composition, understanding the molecular mechanisms that regulate NMDA R subunit expression is of fundamental importance. In the developing cerebellum, there is a switch in the expression of the NMDA R 2B to the 2C subunit during the innervation of granule cells that is accompanied by a change in the channel properties of the receptor. In order to begin understanding the complex mechanisms that regulate the neural-specific expression of NMDA R genes during neurogenesis and modulate their levels in response to synaptic activity, Drs. Michael Sasner and A. Buonanno are investigating the mechanisms that control expression of the 2B and 2C subunits at the transcriptional level.

Studies from our group and others indicate that activity is important for the down-regulation of the NMDA R 2B subunit, and we have recently found that neurotrophic factors and activity contribute to the up-regulation of the 2C gene. Analysis of the 2B gene in transgenic mice reveal that distinct DNA regulatory sequences are required for the neural-specific and developmental down-regulation of the gene. Experiments is transfected primary granule cell neurons are being used to identify the relevant DNA elements and transcription factors conferring these types of regulation. Pharmacological experiments are used to dissociate the pathways required for NMDA R 2C transcriptional activation. In addition, gene knock-out experiments are in progress to determine the significance of the 2B to 2C subunit exchange with respect to cerebellar circuitry and function.

Unit on Molecular Control of Neurodifferentiation

Molecular Basis of Phenotypic Differentiation in the Developing Mammalian Central Nervous System

Using the enkephalin phenotype as a model, the goal of the Unit on Molecular Control of Neurodifferentiation, headed by Dr. Denes v. Agoston, is to understand the molecular process by which the embryonic, multi-potent neuronal stem cell becomes terminally-differentiated and committed to its final neurotransmitter phenotype. Neurons in the CNS communicate with each other and other target cells by utilizing one of the approximately 10 "classical" and one of approximately 30 "peptide" neurotransmitters such as enkephalin. As more and more evidence points toward the developmental origin of major neuropsychiatric disorders, it has become imperative to understand fundamental developmental processes that lead to the normal, balanced phenotypic diversity of the adult CNS. The basal ganglia has been the target of intensive research as a wide array of techniques - from molecular genetics to PET scan - demonstrated that it is the primary "locus morbis" of many mental diseases (autism, schizophrenia, Huntington's and Parkinson's diseases, etc.). The main peptide neurotransmitter of the basal ganglia circuit is enkephalin and its involvement in mediating pathological conditions such as autistic behavior, hallucinations in schizophrenia, personality changes in Huntington's disease, etc. has become more and more evident. Enkephalin, however, is not only a peptide neurotransmitter, but also acts - along with other neuropeptides like VIP - as a critical extracellular signaling molecule during development and disease.

We have been identifying novel DNA elements and their binding proteins that are critical for the developmentally regulated expression of the ENK gene. We also study the role of far-distant DNA interactions and structural changes in the nuclear architecture during terminal neuronal differentiation and search for novel extra- and intracellular signaling mechanisms in the developing mammalian CNS. Our working hypothesis is that extracellular signals act as specifiers for any given phenotype. This early specification is followed by a whole cascade of intracellular events (execution) that result in the spatially restricted expression of the enkephalin gene to a specific subset of neurons characteristic to the adult brain. Based on our preliminary results, this regulation is a progressive restriction of the ENK gene expression which involves multiple interactions between various regulatory elements of the gene and DNA binding proteins that are expressed in a spatially and temporally precisely defined manner in the developing CNS.

We have developed a unique and unorthodox approach ("sample and probe") by combining microdissection of the developing brain regions with powerful molecular biological techniques which permit us to study this developmental regulation at the level of protein-DNA interactions and nuclear structure and provide an unparalleled spatio-temporal resolution at the molecular level. Using this approach, we have identified several novel brain- and development-specific nuclear proteins of which three have been investigated in detail. Three novel proteins bind the truncated octamer [(A/Genk)TTTGCAT=sept] motif located at -542 relative to the transcriptional start site of the rat ENK gene. Stem-sept is expressed in all brain regions between E10 and E14 but the temporal expression of this protein strictly follows the maturation gradient of the various brain regions (spinal cord first, cerebellum last). Stem-sept is down regulated at E16 which is paralleled with the expression of two distinct binding proteins, neuro-sept and glia-sept whose appearance is paralleled with the emergence of neuronal and glial lineages which also follows the maturation gradient of the different brain regions.

In collaboration with Dr. McKay, we established that multipotent neuroglial stem cells grown in culture under proliferating conditions express both the neuro- as well as the glia-sept. However, following changes in culture conditions that induce differentiation into the glial lineage, as indicated by the expression of the glia specific marker GFAP, neuro-sept expression is down-regulated and only glia-sept expression remains detectable. Also, only glia-sept but not neuro-sept is expressed in primary cultures of cortical astroglia. Stem-sept does not require to be phosphorylated as suggested by different dephosphorylation experiments and it can be competed only with the septamer motif, but not with the octamer one. By contrast, both neuro-sept and glia-sept require to be phosphorylated for DNA binding which suggests a potential regulatory mechanism and they can be competed equally with both septamer and octamer motifs. None of the proteins are recognized by any of the existing antibodies raised against various octamer-binding proteins suggesting that these proteins are novel. The binding of the various sept-proteins to the DNA results in a characteristic DNA bending of seemingly different degrees. The temporo-spatial expression pattern of proteins suggests that they may contribute to early neurodifferentiation events of neuronal and glial lineages which precede phenotypic differentiation and the observed DNA bending may play an important role in this process.

Because major cellular differentiation events require interactions between proteins and far-distant DNA elements with consequent alterations in the 3 dimensional arrangement of regulatory elements and because Ca^{2+}-induced DNA sliding may be the mechanism through which these interactions take place, we have continued to study far-distant DNA interactions and changes in the nuclear architecture during neuronal differentiation.

A protein complex that binds in a strict spatio-temporal manner to both the far-upstream (-453 bp) AT-rich region [(ATT)19 repeat] and to the proximal regulatory /TATA region of the ENK gene has been identified. The formed DNA-protein complex is resistant against 2.5 M urea, 2 % NP-40, 14 mM B-mercapto ethanol, 10 mM BAPTA and 1.2 M KCl and to enter PAGE gels. Treatment with 0.001% SDS eliminates DNA binding, and the complex also binds with high affinity to cationic lipids. The DNA binding was found to be Ca^{2+}-dependent and required at least partially phosphorylated protein(s). Competitive mobility shift assays with various mutants of the ATT motif indicated that the protein complex requires a specific DNA structure characterized by AT rich sequences characteristic of matrix attachment regions (MARs) rather than to specific nucleotide sequences typical of conventional transcriptional factors. The protein complex seems to bind to the DNA in an all or nothing fashion, as evidenced by protein titrations suggesting cooperative binding between proteins or between the protein subunits. The length of the repeat was another factor in the strength of the bond. As the length of the oligonucleotide became shorter, the protein became less tightly bound to the DNA, implying a possible binding multimer that is cooperatively bound, but separated by a non-bound DNA section. This is a common pattern for matrix proteins.

Dialysis analysis indicated that the size of the protein complex is approximately 350 kd. UV crosslinking and DNA affinity purification showed that the complex is formed by four proteins of MW ~200 kd, ~ 90 kd, ~ 40 kd and ~14 kd. The 14 kd protein is selectively enhanced in nuclear matrix preparations and forms a high mobility protein-DNA complex with the AT rich motif. However, the most abundant protein of the complex is the ~43 kd protein as indicated by high enrichment following DNA affinity purification.

The very narrow spatio-temporal expression pattern of this ATT-protein complex in the developing brain suggested a neuronal cell type- and developmental-specific repressor role. This hypothesis was supported by *in vivo* experiments using the drug distamycin that specifically binds to the AT-rich minor groove of the DNA and thereby perturbs minor groove-protein interactions typical of nuclear matrix proteins. At the age of E20 when the ATT-binding protein complex is being expressed, distamycin treatment of primary cortical neurons resulted in a ~15-fold increase in ENKmRNA. This finding further supports the proposed repressor role for the complex and for the first time indicates the involvement of the nuclear matrix in phenotypic differentiation of the CNS.

Unit on Neurocytology and Physiology

Regulation of Gene Expression by Neural Impulse Activity

The Unit on Neurocytology and Physiology, directed by **Douglas Fields**, is investigating how expression of genes known to regulate proteins that are important in development of the nervous system (cell adhesion molecules, voltage-gated calcium channels, and immediate early genes), can be regulated by specific patterns of neuronal impulses in neurons, and thereby provide an important influence on the developing structure and function of the nervous system. Effort is now focused on two related areas: (1) identifying important biological consequences of activity-dependent regulation of these genes, and (2) identifying the signaling mechanisms responsible for regulating gene expression by specific patterns of membrane depolarization.

Regulation of Neural Cell Adhesion Molecules by Specific Patterns of Neural Impulses

Specific cell adhesion molecules (CAMs), expressed selectively on different neurons, regulate many of the major processes in development of the nervous system, including cell recognition, aggregation, motility, neurite outgrowth fasciculation and synaptogenesis. Although the expression of distinct neural cell adhesion molecules is regulated precisely during development of the nervous system, our research has shown that expression of the cell adhesion molecule L1 is also controlled by electrical impulse activity in mouse DRG neurons *in vitro*. Correlation of stimulus patterns studied *in vitro* with activity patterns *in utero* suggest that this mechanism could help coordinate the developing structure and function of the nervous system.

In the past year we have found that different CAMs are regulated by different patterns of neural impulses. In DRG neurons, NCAM is not regulated at the mRNA or protein level by frequencies of impulses that affect L1 expression, but a member of the calcium-dependent class of CAMs, N-cadherin, is down regulated by impulse activity of higher frequencies than those that will affect L1 expression. The time-course of activity-dependent regulation has been determined *in vitro* and *in vivo* for the L1 gene, and we have found that L1 returns to normal levels after terminating impulse activity in cell culture. Levels of L1 mRNA increase between embryonic day 11 (E-11) and E-14 in mouse DRG, but levels decrease sharply after E-15. This decreased expression coincides with the time when spontaneous impulse activity, similar to that which lowers L1 expression in culture, begins in these neurons *in vivo*.

We have determined that there are significant functional consequences of activity-dependent regulation of the L1 gene that could affect nervous system development through effects on cell-cell adhesion and axonal fasciculation. Involvement of L1 in axonal-glial association has led us to investigate the possibility that neural impulse activity could regulate neuronal-glial interactions dependent on L1. Brd-U labeling of mitotically active Schwann cells in co-cultures of DRG neurons shows that proliferation of Schwann cells is reduced on axons stimulated at a frequency that lowers L1 expression. Axon myelination is an important developmental process relevant to nervous system disease, and we have developed an *in vitro* assay of axon myelination and used biochemical markers (Gal-C and MAG) and morphological criteria to investigate differences in Schwann cell myelination of DRG axons stimulated to fire action potentials at different patterns in culture.

Other research has shown important interactions between neurotrophins, electrical activity, and cell adhesion molecules. We have determined that specific neurotrophins can prevent the activity-dependent regulation of L1, and we are using specific inhibitors of the Trk receptors to determine which receptors and pathways are involved. Initial studies indicate that the effects of neural impulse activity vary depending upon whether the neuron has or has not innervated appropriate target structures. For instance, L1 expression is not influenced by electrical activity in DRG neurons that have innervated appropriate neurons in the ventral spinal cord. Present evidence suggests that synaptogenesis with the target is not required for this inhibition, but rather that this regulation is mediated by a diffusible signaling molecule secreted by target neurons.

Intracellular Signaling from Neural Impulses

We are monitoring the temporal dynamics of key elements (second messengers, protein kinases, transcription factors) that regulate expression of the immediate early gene *c-fos* in response to membrane depolarization in an effort to understand how intracellular signaling pathways integrate and transmit information coded in the temporal pattern of neural impulses. Results of these studies are consistent with the idea that the differing kinetics of parallel intracellular signaling reactions confers sensitivity to different temporal patterns of activation and thereby activates distinct intracellular pathways controlling gene expression or other neuronal and synaptic functions. We find that transcription of the immediate early gene *c-fos* is sensitive to the pattern of action potential stimulation, and correlated inversely with the interval between successive bursts of action potentials when stimulus bursts are separated by intervals of a few minutes. We do not find that the concentration of any one element in the signaling cascade accounts for the transcriptional regulation in response to different frequencies of activation; instead, the time-dependent concentration dynamics of different elements in the cascade contribute to the transcriptional response to different frequencies of stimulation. For example, the interval between calcium transients correlates with *c-fos* expression, but the amplitude of the calcium transient does not. Phosphorylation of the transcription factor CREB at serine 133 parallels the rapid rate of increase in $[Ca^{++}]_i$, but $[Ca^{++}]_i$ recovers to basal levels much faster than CREB dephosphorylation. For some temporal patterns of stimulation, phosphorylation of CREB at serine 133 was not well correlated with *c-fos* expression. Although CREB is a key regulator of *c-fos* transcription, the results suggest that CREB phosphorylation at serine 133 need not be the determining factor in response to certain patterns of neural impulses.

Unit on Synapse Development and Plasticity

The Unit on Synapse Development and Plasticity, led by Dr. Bai Lu, investigates the molecular mechanisms that govern the formation of specific synapses in the nervous system. Studies are focused on two closely related areas: 1) To determine the role of neurotrophic factors in synapse development and function. 2) To isolate and characterize new molecules that regulate synaptogenesis. Neurotrophic factors are defined as secretory proteins that regulate long-term survival and differentiation of neurons. The role of neurotrophic factors in the structural integrity of the nervous system makes them attractive candidates as therapeutical agents for neurodegenerative diseases. However, recent studies by this group and other laboratories have revealed some unexpected, novel functions of neurotrophic factors in synapse development and plasticity. Of particular significance is our finding that the neurotrophin BDNF regulates the development of long-term potentiation (LTP), a cellular mechanism for learning and memory. In addition, this group continues to characterize molecules involved in specific stages of synaptogenesis. These molecules are potential targets of neurotrophic regulation.

Neurotrophic regulation of the development of long-term potentiation

In the previous year we reported that neurotrophins promote synaptic maturation at the neuromuscular junction and enhance neuronal activity in cultured cortical neurons. To determine the physiological significance of neurotrophic modulation of neurotransmitter release in the brain, particularly with regard to synapse development, we studied the effect of BDNF on hippocampal LTP. The ability of the hippocampus to undergo LTP induced by tetanic stimulation increases with age, paralleling the expression of BDNF and its receptor TrkB. Exogenous BDNF promoted the induction of LTP in young (postnatal day 12-13) hippocampal slices, which in the absence of BDNF show only short-term potentiation. In contrast, a TrkB-IgG fusion protein, which scavenges endogenous BDNF, reduced the magnitude of LTP in adult hippocampus. Thus, a critical level of BDNF/TrkB activity appears to be important for long-term modulation of synaptic efficacy. Exogenous BDNF promoted synaptic responses to tetanus and enhanced LTP in the neonatal hippocampus where endogenous BDNF level is low, while TrkB-IgG had opposite effects in adult hippocampus where endogenous BDNF level is high. The effect of BDNF on LTP development was due to an enhanced ability of hippocampal synapses to respond to tetanic stimulation, rather than to a direct modulation of the LTP-triggering mechanism. Our results suggest that BDNF may play an important role in long-term synaptic plasticity such as LTP, and may provide insights into molecular mechanisms for the activity-dependent regulation of synaptogenesis and plasticity.

Characterization of molecules involved in synapse development and plasticity

Our previous experiments indicated that 1) loading of exogenous synapsin I, a synaptic vesicle protein, to the nerve terminals speeds up the maturation of the neuromuscular synapse, and 2) neurotrophic regulation of synapse maturation may be mediated, at least in part, by increasing synapsin I expression. We now have evidence that functional maturation of synapses may depend upon the expression of synapsin I at the presynaptic terminals. Synapsin I can be used as a biochemical marker for synapse maturation both *in vivo* and in culture, because its expression correlates precisely with the maturation process. When endogenous synapsin I was induced to express earlier, synapse maturation occurred earlier. Another molecule we identified is the Ca^{2+}-binding protein frequenin. We have previously shown that frequenin facilitates neurotransmitter release at *Xenopus* neuromuscular synapses. In Drosophila, frequenin has been shown to potentiate synaptic responses to high frequency stimulation. To determine its cellular and subcellular localization as well as its potential function in the mammalian brain, we cloned mouse frequenin (Mfreq) and characterized its expression both *in vivo* and in culture. An antibody raised against frequenin detect a 24 kDa protein on SDS gel. Mfreq mRNA and protein are widely distributed in the brain, spinal cord, and dorsal root ganglia, but not outside the nervous system. *In situ* hybridization and immunocytochemistry demonstrated that Mfreq is expressed primarily in neurons. Double immunofluorescence microscopy established that Mfreq was co-localized with the synapse marker, SV2 in cultured hippocampal neurons. We are currently investigating whether frequenin is involved in BDNF regulation of synaptic responses to tetanic stimulation in the hippocampus.

LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

ANNUAL BIBLIOGRAPHY

Agoston Dv, Palkovits CG, Fitzgerald SF, Brenneman DE. Developmental changes in the inducibility of fos-like immunoreactivity in primary embryonic spinal cord cultures. Dev Brain Res 1995;89:173-86.

Baler R, Coon SL, Klein DC. Orphan nuclear receptor RZRβ: Cyclic AMP regulates expression in the pineal gland, Biochem Biophys Res Commun 1996;220:975-8.

Baler R, Klein DC. Circadian expression of transcription factor Fra 2 in the rat pineal gland, J Biol Chem 1995;270:27319-25.

Baler R, Klein DC. Circadian regulation of transcription factor FRA-2 in the rat pineal gland. In: Advances in Pineal Res, 1996; Vol 9 (in press).

Bamberger AM, Bamberger CM, Puy LA, Asa SL. Regulation of the GH gene family in placenta and pituitary. In: De Bellis A and Schipani E, eds. Future trends in endocrinology, Serono Symposia Series, Frontiers in Endocrinology 1995;Vol 14, pp 77-82.

Bamberger AM, Pu L-P, Cool DR, Loh YP. The neuro-2A cell line expresses [met]-enkephalin and vasopressin mRNA and peptide. Mol and Cell Endocrinol 1995;113:155-63.

Bernard M, Donohue SJ, Klein DC. Human hydroxyindole-O-methyltransferase in pineal gland, retina and Y-79 retinoblastoma cells, Brain Res 1995;696:37-48.

Bernard M, Iuvone PM, Cassone VM, Roseboom PH, Coon SL, Klein DC. Chicken arylalkylamine-N-acetyltransferase: Cloning and circadian regulation in the pineal gland. In: Advances in Pineal Research, 1996; Vol 9 (in press).

Bernard M, Iuvone PM, Cassone VM, Roseboom PH, Coon SL, Klein DC Melatonin synthesis: Photic and circadian regulation of serotonin N-acetyltransferase mRNA in the chicken pineal gland and retina, J Neurochem (in press).

Bernard M, Klein DC. Melatonin synthetic enzyme: Retinoic acid increases hydroxyindole-O-methyltransferase activity and mRNA in human Y-79 retinoblastoma cells, J Neurochem (in press).

Bernard M, Voisin P, Klein DC. Hydroxyindole-O-methyltransferase in Y-79 cells: Regulation by serum, Brain Res 1996;727:118-24.

Brenneman DE, Gozes I. A femtomolar acting neuroprotective peptide. J Clin Invest 1996;97:2299-2307.

Brenneman DE, Phillips TM, Festoff BW, Gozes I. Identity of neurotrophic molecules released from astroglia by vasoactive intestinal peptide. Annls NY Acad Sci, in press.

Calvo S, Stauffer J, Nakayama M, Buonanno A. Transcriptional control of muscle plasticity: differential regulation of troponin I genes by electrical activity. Dev Genetics, in press.

Cawley NX, Pu L-P, Loh YP. Immunological identification and localization of yeast aspartic protease 3-like prohormone processing enzymes in mammalian brain and pituitary. Endocrinology in press.

Cool DR, Louie DY, Loh YP. Yeast aspartic protease 3 (YAP3P) is sorted to secretory granules and activated to process pro-opiomelanocortin in PC12 cells. Endocrinology (in press).

Coon SL, Mazuruk K, Bernard B, Roseboom PH, Klein DC, Rodriguez IR. The human serotonin *N*-acetyltransferase (EC 2.3.1.87) Gene: Structure, chromosomal localization and tissue expression, Genomics 1996;34:76-84.

Coon SL, Roseboom PH, Baler R, Weller JL, Namboodiri MAA, Koonin EV, Klein DC. Pineal serotonin N-acetyltransferase (EC 2.3.1.87): Expression cloning and molecular analysis, Science 1995;270:1681-83.

Davenport RW, Ping D, Mills LR, Kater SB. Distinct calcium signaling within neuronal growth cones and filopodia. J Neurobiol 1996;31:1-15.

Davenport RW, Thies E, Nelson PG. Cellular localization of guidance cues in the establishment of retinotectal topography. J Neurosci 1996;16:2074-85.

Erickson J, Weihe E, Shäefer MK-M, Neale EA, Williamson L, Bonner TI, Tao-Cheng SJ-H, Eiden LE. The VACh/ChAT "cholinergic gene locus": new aspects of genetic and vesicular regulation of cholinergic function. In: Cuello CA, ed. Progr Brain Res, Amsterdam: Elsevier, in press.

Festoff BW, Nelson PG, Brenneman DE. Prevention of activity-dependent neuronal death: vasoactive intestinal polypeptide stimulates astrocytes to secrete the thrombin-inhibiting neurotrophic serpin, protease Nexin I. J Neurobiol 1996;30:255-66.

Fields RD. Signaling from neural impulses to genes. The Neuroscientist, in press.

Fields RD, Itoh K. Neural cell adhesion molecules in activity-dependent development and synaptic plasticity. Trends Neurosci, in press.

Figourov A, Pozzo Miller L, Wang T, Olafsson P, Lu B. Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 1996;381: 706-9.

Friedman TC, Cool DR, Jayasvasti V, Louie D, Loh YP. Processing of pro-opiomelanocortin in GH3 cells: Inhibition by prohormone convertase 2 (PC2) antisense mRNA. Mol and Cell Endocrinol 1996;116:89-96.

Friedman TC, Loh YP, Cawley NX, Birch NP, Huang SS, Jackson IMD, Nillni EA. Processing of pro-TRH by bovine intermediate lobe secretory vesicle membrane PC1 and PC2 enzymes. Endocrinology 1995;136:4462-72.

Friedman TC, Mastorakos G, Bouzas EA, Newman TD, Mullen NM, Horton EG, Costello R, Papadopoulos NM and Chrousos GP. Carbohydrate and lipid metabolism in endogenous hypercortisolism: Shared features and metabolic syndrome X and NIDDM. Endocrine J (in press).

Friedman TC, Yanovski JA. Morning plasma free cortisol: Inability to distinguish patients with mild Cushing syndrome from patients with pseudo-cushing states. J Endocrinol Invest 1995;18:696-701.

Friedman TC, Yanovski JA, Nieman LK, Doppman JL, Cutler Jr., GB, Oldfield EH, Chrousos GP, Kalogeras KT. Inferior petrosal sinus arginine vasopressin concentrations in normal volunteers and patients with Cushing disease. J Clin Endocrinol Metab (in press).

Gibney G, Buonanno A. Analysis of neural-responsive myogenin upstream sequences by myoblast implantation. Dev Biol 1995;172: 614-24.

Gozes I, Bardea A, Reshef A, Zamostiatno R, Zhukovsky S, Rubinraut S, Fridkin M, Brenneman D.E. Novel neuroprotective strategy for Alzheimer's disease: inhalation of a fatty neuropeptide. Proc Natl Acad Sci USA 1996;93:427-32.

Gozes I, Brenneman DE. Activity-Dependent Neurotrophic Factor (ADNF): an extracellular neuroprotective Chaperonin? J Mol Neurosci, in press.

Gozes I, Fridkin M, Brenneman DE. A VIP hybrid antagonist: from neurotrophism to clinical applications. Cell Mol Neurobiol 1995;15:675-87.

Gozes I, Fridkin M, Brenneman DE. Stearyl-Nle-VIP: a non-invasive impotence drug and a potent agent of neuroprotection. Drugs of the Future 1995;20(7):680-95.

Gressens P, Paindaveine PB, Hill JM, Brenneman DE, Evrard P. Growth factor properties of VIP during early brain development: whole embryo culture and in vivo studies. Ann NY Acad Sci, in press.

Halpern JL, Neale EA. Neurospecific binding, internalization, and retrograde axonal transport. In: Montecucco C, ed. Clostridial Neurotoxins. The Molecular Pathogenesis of Tetanus and Botulism. Berlin: Springer, 1995;221-41.

Hill JM, Gressens P, Brenneman DE. Growth of the early postimplantation embryo: regulation by high affinity, GTP-insensitive VIP receptors. Annls NY Acad Sci, in press.

Hill RM, Ledgerwood EC, Brennan SO, Pu L-P, Loh YP, Christie DL, Birch NP. Comparison of the molecular forms of the Kex2/subtilisin-like serine proteases SPC2, SPC3 and furin in neuroendocrine secretory vesicles reveals differences in carboxyl terminus truncation and membrane association. J Neurochem 1995;65:2318-26.

Hill JM, McCune SK, Alvero RJ, Glazner GW, Brenneman DE. VIP regulation of embryonic growth. Ann NY Acad Sci, in press.

Hill JM, McCune SK, Alvero RJ, Glazner G, Henins K, Stanziale SF, Keimowitz JR, Brenneman DE. Maternal vasoactive intestinal peptide and the regulation of embryonic growth in the

rodent. J Clin Invest 1996;97:202-8.

Klein DC, Coon SL, Mazuruk K, Bernard M, Roseboom PH, Rodriguez IR. Biological rhythms and psychiatric disease: Focus on melatonin-related genes. In: Wahlstrom J,Wetterberg L, eds. Genetics and Psychiatric Disorders. Oxford: Elsevier Science (in press).

Klein DC, Roseboom PH, Coon SL. New light is shining on the melatonin rhythm enzyme: The first post cloning view, Trends in Endocrinol Metabol 1996;7:106-12.

Kuenzel WJ, McCune SK, Talbot RT, Sharp PJ, Hill JM. Sites of gene expression for vasoactive intestinal peptide (VIP) throughout the brain of the chick (Gallus domesticus). J Comp Neurol, in press.

Ledgerwood EC, Brennan SO, Cawley NX, Loh YP, George PM. Yeast aspartic protease 3 (YAP3) prefers substrates with basic residues in the P_2, P_1 and P_2' positions. FEBS Lett 1996;383:67-71.

Li MX, Jia M, Fields RD, Nelson PG. Modulation of calcium currents by electrical activity. J Neurophysiol, in press.

Lilling G, Wollman Y, Goldstein MN, Rubinraut S, Fridkin M, Brenneman DE, Gozes I. Inhibition of human neuroblastoma growth by a specific VIP antagonist. J Mol Neurosci 1995;5:231-9.

Lipton, S.A., Brenneman, D.E., Silverstein, F.S., Masliah, E. And Mucke, L. Gp120 and neurotoxicity *in vivo*. Trends Pharmacol Sci 1995;16:122.

Loh YP, Snell CR, Cool DR. Evidence for a receptor-mediated mechanism for sorting pro-opiomelanocortin to the regulated secretory pathway. In: Krisch, B, ed. Advances in Life Sciences; Birkhäuser Press, Switzerland (in press).

Lu B, Papov S, Czernik A, Wang T, Poo M-M, Greengard P. Expression of synapsin I reflects maturation of neuromuscular synapse, Neurosci, in press.

McCune SK, Hill JM. Ontogenic expression of two alpha-one adrenergic receptor subtypes in the rat brain. J Mol Neurosci 1995;6:51-62.

Nakayama M, Stauffer J, Basu S, Wawrousek E, Buonanno A. Common core elements are found in skeletal muscle slow and fast fiber-type specific enhancers. Mol Cell Biol 1996;16:2408-17.

Neale EA, Bowers LM, Williamson LC. Vesicle membrane traffic at the synaptic terminal. In: Foster KA, ed. Biomedical Aspects of Clostridial Neurotoxins, in press.

Nillni EA, Friedman TC, Todd RB, Birch NP, Loh YP, Jackson IMD. ProTRH processing by PC1. J Neurochem 1995;65:2462-72.

Olafsson P, Soares HD, Wang T, Herzog K-H, Morgan JI, Lu B. The Ca2^{+} binding protein frequenin is a nervous system-specific protein preferentially localized in neurites. Mol Brain Res, in press.

Pu L-P, Ma W, Barker JL, Loh YP. Differential co-expression of genes encoding pro-TRH and prohormone convertases (PC1 and PC2) in rat brain neurons: Implications for differential processing of pro-TRH. Endocrinology 1996;137:1233-41.

Pu L-P, Van Leeuwen FW, Tracer HL, Sonnemans MAF, Loh YP. Localization of Vasopressin mRNA and immunoreactivity in pituicytes of pituitary stalk-transected rats after osmotic stimulation. Proc Natl Acad Sci USA 1995;92:10653-57.

Roseboom PH, Coon SL, Baler R, McCune SK, Weller JL, Klein DC. Melatonin synthesis: Regulation of the more than 150-fold nocturnal increase in serotonin N-acetyltransferase messenger RNA in the rat pineal gland. Endocrinology 1996;137:3033-44.

Rosenthal, N. and Buonanno, A. (1996) Molecular mechanisms regulating muscle plasticity. Dev Genetics, in press.

Sasner M, Buonanno A. Distinct NMDA 2B subunit gene sequences confer neural and developmental specific expression. J Biol Chem, in press.

Weller JL, Jaffee H, Roseboom PH, Zylka M, Klein DC. 2D-PAGE analysis: Adrenergically regulated pineal protein AIP 37/6 is a phosphorylated isoform of cytosolic malate dehydrogenase, Brain Res 1996;713:8-16.

Williamson LC, Halpern JL. Clostridial neurotoxin action in spinal cord neurons after uptake by an alternate pathway. In: Foster KA, ed. Biomedical Aspects of Clostridial Neurotoxins, in press.

Williamson LC, Halpern J, Montecucco C, Brown JE, Neale EA. Clostridial neurotoxins and substrate proteolysis in intact neurons: Botulinum neurotoxin C acts on synaptosomal protein of 25 kDa. J Biol Chem 1996;271: 7694-9.

LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

ANNUAL REPORT STAFF LISTING

October 1, 1995 through September 30, 1996

Chief

*Phillip G. Nelson, M.D., Ph.D.

Senior Staff
Douglas E. Brenneman, Ph.D.
Joanna Hill, Ph.D.
David C. Klein, Ph.D.
Y. Peng Loh, Ph.D.
Elaine A. Neale, Ph.D.

Expert
Andrew Parfitt, Ph.D.

Fellows
Andres Buonanno, Ph.D.
Raquel Castellon, Ph.D.
Niamh Cawley, Ph.D.
David Cool, Ph.D.
Steven L. Coon, Ph.D.
R. Douglas Fields, Ph.D.
Jonathan Gastel, Ph.D.
Gretchen Gibney, Ph.D.
Gordon W. Glazner, Ph.D.
Sunghee Kim, Ph.D.
Susan Lee, Ph.D.
Bai Lu, Ph.D.
Alvin Lyckman, Ph.D.
Patrick Roseboom, Ph.D.
Michael J. Sasner, Ph.D.
Fu-Sheng Shen, Ph.D.
Jimmy Stauffer, Ph.D.
Lura C. Williamson, Ph.D.

Visiting Program
Denes v. Agoston, M.D., Ph.D.
Marianne Bernard, Ph.D.
Albert Dobi, Ph.D.
Feleke Eshete, Ph.D.
Alexandre Figourov, M.D.
Min Jia, M.D.
Francis Lim, Ph.D.
Keiko Mizuno, Ph.D.
Emmanuel Normant, Ph.D.
Chikara Oho, Ph.D.

Intergovernmental Personnel Act (IPA)
Peter Latham, Ph.D.

Guest Researcher
Rueben Baler, Ph.D.
Soledad Calvo, M.D., Ph.D.
Roger Davenport, Ph.D.
Illana Gozes, Ph.D.
George Kunos, M.D., Ph.D.
Min-Xu Li, M.D.
Qun Li, M.D., Ph.D.
Susan McCune, M.D.
M.A. Aryan Namboodiri, Ph.D
Chunfa Zhang, Ph.D.

Project Numbers - Annual Report 1996

LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY (LDN)

ZO1 HD 00047-27	Biochemical Studies of Neurons and Other Cell Types Douglas E. Brenneman, Ph.D.
ZO1 HD 00056-21	Biosynthesis, Processing and Secretion of Neuropeptides and Pituitary Peptide Hormones Yoke Peng Loh, Ph.D.
ZO1 HD 00064-20	Neurobiologic Studies of Neurons and Glia in Cell Culture Phillip G. Nelson, M.D., Ph.D.
ZO1 HD 00094-25 **TERMINATED**	Pineal Regulation: Environmental and Physiology Factors David C. Klein, Ph.D.
ZO1 HD 00095-26	Pineal Regulation: Transsynaptic and Intracellular Mechanisms David C. Klein, Ph.D.
ZO1 HD 00704-12	Tetanus Toxin Effects and Localization in Neurons Elaine A. Neale, Ph.D.
ZO1 HD 00708-12	Morphological Studies of Neuronal and Non-Neuronal Cells in CNS Cell Cultures Elaine A. Neale, Ph.D.
Z01 HD 00710-08	Molecular Characterization of Glutamate Receptor Expression in Brain Andres Buonanno, Ph.D.
Z01 HD 00711-07	Transcriptional Regulation of Skeletal Muscle-Specific Genes by Electrical Activity Andres Buonanno, Ph.D.
Z01 HD 00712-05	Regulation of Phenotypic Differentiation in the Developing Mammalian CNS Denes Agoston, M.D.
Z01 HD 00713-02	Regulation of Gene Transcription and Neurite Outgrowth by Neural Impulse Activity Richard D. Fields, Ph.D.
Z01 HD 00714-01	Molecular Mechanisms of Synapse Development and Plasticity Bai Lu, Ph.D.

Z01 HD 00715-799

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00047-27 LDN

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Studies of Neurons and Other Cell Types

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: D. Brenneman

Others:	J. Hill	Staff Scientist	LDN, NICHD
	G. Glazner	IRTA Fellow	LDN, NICHD
	G. Gibney	IRTA Fellow	LDN, NICHD
	S. Lee	PRAT Fellow	LDN, NICHD
	I. Gozes	Guest Researcher	LDN, NICHD
	S. Servoss	Guest Researcher	LDN, NICHD
	J. Hauser	Biologist	LDN, NICHD

COOPERATING UNITS *(if any)* Dept. Organic Chem., Weizmann Inst. (M. Fridkin); Dept. Neurol., Univ. Kansas (B. Festoff); Dept. of Immunol. George Washington Univ. (T. Phillips); Dept. Surgery, Univ. Queensland (A. Cavanagh); Dept. Pediatrics, Johns Hopkins Univ. (S. McCune).

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Developmental and Molecular Pharmacology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	7.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Expression cloning studies directed at Activity Dependent Neurotrophic Factor (ADNF) were conducted using neutralizing antiserum derived from determined peptides of biochemically isolated ADNF. A positive clone (CL25) was obtained from a library of P19 mouse embryonic carcinoma cell line. The cDNA for CL25 had a unique sequence that contained 2358 base pairs with an open reading frame of 811 amino acids. A fusion protein from this clone increased neuronal survival of cerebral cortical cultures treated with tetrodotoxin. The neuroprotective activity of the fusion protein was partially blocked by co-treatment with neutralizing antiserum from ADNF I. The protein encoded by CL25 was named ADNF 3 because of its similar biological activity and homologous structure to ADNF I-derived peptides. An active peptide fragment of CL25 was synthesized and determined to be neuroprotective against beta amyloid peptide. The CL25 peptide exhibited a bi-modal dose response. Another positive clone (CL8) was obtained by functional screening of a library of human astrocytoma. The cDNA had unique nucleotide sequence with high identity to that of known peptide sequences derived from ADNF I. The fusion protein from CL8 prevented cell death associated with electrical blockade in cerebral cortical cultures, an effect completely blocked by antiserum to ADNF I. The protein encoded by CL8 appears to be a human homolog of ADNFI and was named ADNF 4. A sub-femtomolar-acting peptide from CL8 was found to protect neurons in cerebral cortical cultures from cell death associated with tetrodotoxin and gp120, the envelope protein from HIV. Growth-promoting effects of early pregnancy factor (EPF) were observed in embryonic day 9 mice. EPF produced increases in somite number and size that were similar to that previously observed for vasoactive intestinal peptide and ADNF. Neutralizing antiserum to EPF produced significant reduction in somitic growth in comparison to controls. In situ hybridization studies demonstrated that EPF was elevated in embryonic membranes and in the floor plate of the neural tube. Autoradiographic studies of VIP binding also showed high density of sites in the floor plate of the neural tube. VIP-mediated increases in embryonic growth and shortening of cell cycle duration were associated with transcriptional activation of multiple cyclins and *fos*. Insulin-like growth-factor-1 appears to mediate in part VIP-stimulation of embryonic growth.

Project Description

Objectives:

1) To investigate the structure and functions of activity dependent neurotrophic factor, a survival-promoting substance that is released by vasoactive intestinal peptide.

2) To study the relationship between VIP and glia-derived cytokines and protease inhibitors.

3) To extend the investigation of VIP, VIP analogues, ADNF and Early Pregnancy Factor to embryonic development *in utero*.

4) To develop and characterize new agonists and antagonists to VIP receptors in the central nervous system.

5) To study the neurotoxicity of the HIV envelope protein in developing animals and to devise VIP-related protective agents against HIV-related viral products.

Methods Employed:

Dissociated cultures derived from the central nervous system of fetal rodent were used for studies of VIP, cytokines and growth factors that increase neuronal survival. In particular, the development of cerebral cortical cultures from term rats was emphasized. This preparation allowed for a system homologous to that used for the isolation of ADNF. Both neuronal and mixed neuronal plus glia cultures were used to test for neuroprotective and survival-promoting actions. Functional screening of cDNA libraries was implemented with these culture systems. Computerized image analysis, calcein uptake and ouabain binding were used to assess neuronal populations in culture. For specific cellular phenotype assessment, immunocytochemical methods for neuron specific enolase, glial fibrillary acid protein, methionine-enkephalin and vasoactive intestinal peptide were performed. Quantitative Northern blot hybridizations and RT-PCR were conducted for the analysis of neuropeptide, cyclin, growth factor and transcriptional factor gene expression. The following biochemical techniques were used: radioimmunoassay for peptides and cyclic nucleotides, capillary electrophoresis combined with luminescence-based ELISA assays for cytokines, one and two dimensional polyacrylamide gel electrophoresis, assays for choline acetyl transferase and glutamic acid decarboxylase and radioligand binding. Standard chromatographic (anion exchange, sizing, and reverse phase) separations were conducted to purify ADNF and to measure VIP degradation products. Autoradiography of VIP receptor binding was conducted on frozen thin sections of brain as was *in situ* hybridization. Whole embryo cultures and embryos obtained *in utero* were used to study the growth promoting actions of VIP and related drugs. E9.5 mouse embryos were incubated for up to 24 hours in the presence of fresh rat serum. Embryos were evaluated by somite number, RT-PCR gene expression, embryonic volume, protein and DNA content, receptor distribution and by mitotic assay of bromodeoxyuridine incorporation into nuclei as detected by immunocytochemistry. For analysis of dendritic arbor, Golgi impregnation was utilized with quantitative image analyses. For cloning studies of ANDF, expression libraries were generated from P19 mouse, rat astrocytes and a human astrocytoma cell line. Libraries were screened both by antibody and functional assays of fusion proteins. Direct sequencing was performed by automated DNA sequencing.

Major Findings

1. Expression cloning of a P19 library with anti-ADNF 1 resulted in a positive clone (CL25) with a unique nucleotide sequence that contained 2358 base pairs with an open reading frame of 811 amino acids. This discovery was in collaboration with Illana Gozes and Meirav Basan at Tel Aviv University.

2. A fusion protein from CL25 increased neuronal survival in cerebral cortical cultures treated with tetrodotoxin. This neuroprotective effect was partially prevented by co-treatment with antiserum against ADNF I. The protein encoded by CL25 was named ADNF 3 because of its similar biological activity and homologous structure to ADNF I-derived peptides.

3. An active peptide of CL25 was discovered based on a homologous sequence to ADNF-9, a femtomolar acting neuroprotective peptide derived from ADNF 1. Like ADNF-9, the CL25 peptide prevented neuronal cell death associated with beta amyloid peptide, a toxic substance implicated in the etiology of Alzheimer's disease. A bimodal dose response was observed for this protective effect.

4. Screening of a human astrocytoma library with a functional assay of neuroprotection from electrical blockade in cerebral cortical cultures revealed a positive clone (CL8). The cDNA for CL8 had a unique sequence with an open reading frame of 200 base pairs. This discovery was made by Gretchen Gibney.

5. The fusion protein from CL8 prevented cell death associated with tetrodotoxin in cerebral cortical cultures. This neuroprotective action was completely prevented by co-treatment with antiserum against ADNF I. The protein encoded by CL8 appears to be a human homolog to ADNF I and was named ADNF 4.

6. An active peptide of CL8 was discovered based on a homologous sequence to ADNF-9. The peptide of CL8 prevented neuronal cell death in cerebral cortical cultures treated with tetrodotoxin or gp120, the neurotoxic envelope protein from the human immunodeficiency virus.

7. Early Pregnancy Factor (EPF) produced significant increases in somitic number and growth in whole embryo cultures of embryonic day 9 mice. The magnitude of the growth stimulation was similar to that observed previously with vasoactive intestinal peptide or ADNF. Drs. Hill and Lee collaborated on the studies on EPF and embryonic development.

8. Treatment of whole embryo cultures with neutralizing antiserum to EPF produced a decrease in somitic growth and size in comparison to controls.

9. *In situ* hybridization studies demonstrated that mRNA for EPF was evident in extra-embryonic membranes and in the floor plate of the neural tube at embryonic day 9 of mice.

10. Joanna Hill conducted autoradiographic studies of VIP binding which demonstrated high density of specific sites in the floor plate of the neural tube at embryonic day 9. This is highly significant in that this region is the center governing tissue organization.

11. In studies conducted by Steve Servoss and Gordon Glazer, VIP-mediated increases in embryonic growth and cell cycle shortening of neuroepithelial cells were associated with transcriptional activation of multiple cyclins and the early intermediate gene *c-fos*.

12. Investigations of VIP-mediated increases in embryonic growth revealed that IGF-1 appears to act downstream from both VIP and ADNF.

Significance to Biomedical Research and the Program of the Institute.

The discovery of activity dependent neurotrophic factor and its relationship to vasoactive intestinal peptide neurotrophism is a fundamental contribution made by this Section. The basic observation of VIP's neurotrophic action has been extended and given further depth by the recognition of ADNF's role in developmental regulation and neuroprotection. ADNF and related peptides have provided a new concept in neuroprotection: **extracellular stress proteins**. ADNF is a 14 kDalton protein that has sequence similarity to a stress protein (heat shock protein 60), an intracellular molecule that has been recognized to be important in protein folding and cellular protection from thermal stress and toxic substances. Although the mode of action for ADNF is not yet established, the identification of a survival-promoting, extracellular molecule opens a whole new orientation and scope of investigation into the regulation of neuroprotection and neuronal cell survival. With the cloning of mouse and human homologs to ADNF, a family of biologically and structurally related growth factors has begun to emerge. The identification of these molecules should provide a framework for exciting new discoveries of growth regulation and neuroprotection. The role of VIP as a developmental regulator has been significantly advanced with the recognition that multiple cyclins, IGF-1 and c-fos are important intermediaries of VIP-mediated growth regulation of mid-gestational embryos. The only other recognized extracellular stress protein, early pregnancy factor, is also under intense study as it relates to embryonic growth regulation. Together, this Section has provided strong direction to the potential of these extracellular stress proteins as growth regulators. The further investigation of VIP and related molecules actions will surely produce greater insight into the regulation of brain and embryonic development and provide exciting new therapeutic approaches to the treatment of human neurodegenerative disease.

Proposed Course

The ADNF clones (CL25 and CL8) will be used to quantitate and localize mRNA's in both rodent and human brain tissue. In situ hybridization studies will focus also on the distribution of CL25 in the embryonic mouse.

Genomic clones of both CL25 and CL8 will be isolated.

A "knockout mouse" will be generated with the CL25 mouse gene.

Antisera against ADNF will be tested for effects on the development of mammalian embryos. If the anti-serum produces growth retardation, detailed neuroanatomical and biochemical studies will be conducted. Investigations of developmental milestones and learning behavior will also be tested in neonates from mothers treated with anti-ADNF.

In collaboration with our colleagues in Israel, the molecular cloning of other ADNFs will continue to be pursued, particularly in the rat. With the acquisition of the cDNAs to ADNF 3 and 4, other libraries will screened for additional members of this family of proteins.

The relationships between VIP, ADNF and chaparonin-10 will also be explored during mid-gestational pregnancy. Collaborations with Alice Cavanaugh will focus on the role of early pregnancy factor and its potential role in regulating embryonic development.

Effort will intensify on developing a biochemical/immunological assay for ADNF. With the production of large amounts of rabbit anti-ADNF and the procurement of capillary electrophoresis equipment, this goal should see progress.

Animal

93-025	Brenneman	The role of vasoactive intestinal peptide and related peptides in neurobehavior development and the morphology and neurochemistry of the developing nervous system
93-026	Brenneman	The role of vasoactive intestinal peptide and related peptides on behavior and the morphology and neurochemistry of the brain
94-021	Brenneman	Glial-derived trophic factors
95-003	Brenneman	Influence of VIP and antagonists on developing neuroepithelium in mouse embryos
95-034	Brenneman	Influence of growth factors on a transgenic mouse model of amyotrophic lateral sclerosis exhibiting motor neuron disease

Publications

Agoston DV, Palkovits CG, Fitzgerald SF, Brenneman DE. Developmental changes in the inducibility of fos-like immunoreactivity in primary embryonic spinal cord cultures. Dev Brain Res 1995;89:173-86.

Brenneman DE, Gozes I. A femtomolar acting neuroprotective peptide. J Clin Invest 1996;97:2299-2307.

Brenneman DE, Phillips TM, Festoff BW, Gozes I. Identity of neurotrophic molecules released from astroglia by vasoactive intestinal peptide. Annls NY Acad Sci, in press.

Festoff, B.E., Nelson, P.G. and Brenneman ,D.E. Prevention of activity-dependent neuronal death: vasoactive intestinal polypeptide stimulates astrocytes to secret the thrombin-inhibiting, neurotrophic serpin, protease nexin I. J Neurobiol 1996;30:255-66.

Gozes I, Bardea A, Reshef A, Zamostiatno R, Zhukovsky S, Rubinraut S, Fridkin M, Brenneman D.E. Novel neuroprotective strategy for Alzheimer's disease: inhalation of a fatty neuropeptide. Proc Natl Acad Sci USA 1996;93:427-32.

Gozes I, Brenneman DE. Activity-Dependent Neurotrophic Factor (ADNF): an extracellular neuroprotective Chaperonin? J Mol Neurosci, in press.

Gozes I, Fridkin M, Brenneman DE. A VIP hybrid antagonist: from neurotrophism to clinical applications. Cell Mol Neurobiol 1995;15:675-87.

Gozes I, Fridkin M, Brenneman DE. Stearyl-Nle-VIP: a non-invasive impotence drug and a potent agent of neuroprotection. Drugs of the Future 1995;20(7):680-95.

Gressens P, Paindaveine PB, Hill JM, Brenneman DE, Evrard P. Growth factor properties of VIP during early brain development: whole embryo culture and in vivo studies. Ann NY Acad Sci, in press.

Hill JM, Gressens P, Brenneman DE. Growth of the early postimplantation embryo: regulation by high affinity, GTP-insensitive VIP receptors. Annls NY Acad Sci, in press.

Hill JM, McCune SK, Alvero RJ, Glazner GW, Brenneman DE. VIP regulation of embryonic growth. Ann NY Acad Sci, in press.

Hill JM, McCune SK, Alvero RJ, Glazner G, Henins K, Stanziale SF, Keimowitz JR, Brenneman DE. Maternal vasoactive intestinal peptide and the regulation of embryonic growth in the rodent. J Clin Invest 1996;97:202-8.

Kuenzel WJ, McCune SK, Talbot RT, Sharp PJ, Hill JM. Sites of gene expression for vasoactive intestinal peptide (VIP) throughout the brain of the chick (Gallus domesticus). J Comp Neurol, in press.

Lilling G, Wollman Y, Goldstein MN, Rubinraut S, Fridkin M, Brenneman DE, Gozes I. Inhibition of human neuroblastoma growth by a specific VIP antagonist. J Mol Neurosci 1995;5:231-9.

Lipton, S.A., Brenneman, D.E., Silverstein, F.S., Masliah, E. And Mucke, L. Gp120 and neurotoxicity *in vivo*. Trends Pharmacol Sci 1995;16:122.

McCune SK, Hill JM. Ontogenic expression of two alpha-one adrenergic receptor subtypes in the rat brain. J Mol Neurosci 1995;6:51-62.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00056-21 LDN

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biosynthesis, processing & secretion of neuropeptides & pituitary peptide hormones

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and Institute affiliation)*

PI:	Y. Peng Loh	Section Chief	LDN, NICHD
Others:	LePing Pu	Visiting Fellow	LDN, NICHD
	David Cool	IRTA	LDN, NICHD
	Niamh Cawley	IRTA	LDN, NICHD
	Emmanuel Normant	Visiting Fellow	LDN, NICHD
	ChunFa Zhang	Courtesy Assoc	LDN, NICHD
	Vicki Olsen	Guest Researcher	LDN, NICHD
	FuSheng Shen	IRTA	LDN, NICHD
	Winnie Tam	Microbiologist	LDN, NICHD

COOPERATING UNITS *(if any)*
ERRB (H-C Chen); U of Auckland, New Zealand (N. Birch); Cambridge University, England (T. Blundell)
Sandoz Inst. Med. Res. London (C. Snell); NIDDK (L. Parnell)

LAB/BRANCH Laboratory of Developmental Neurobiology

SECTION Section on Cellular Neurobiology

INSTITUTE AND LOCATION NICHD, NIH, Bethesda, MD

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
8	5.5	2.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The sorting signal motif for targeting pro-opiomelanocortin (POMC, pro-ACTH/endorphin) and pro-enkephalin (pro-Enk) to the regulated secretory pathway was identified. For POMC, the motif consists of a 13 amino acid amphipathic loop stabilized by one disulfide bridge located at the N-terminus of POMC. Two hydrophobic and two acidic residues were on the surface of this loop and mutagenesis studies showed that they were important for the sorting function of the signal. A similar 19 amino acid amphipathic loop sorting signal was also identified at the N-terminus of pro-Enk, indicating that these motifs represent consensus sorting signals. A sorting receptor that is specific for the sorting signal of POMC has been identified in bovine pituitary Golgi and secretory granule membranes. Binding studies showed that other regulated pathway proproteins e.g. pro-Enk, pro-insulin and chromogranin A also bound to the receptor and were displaced by N-POMC1-26 which contains the POMC sorting signal motif. However, constitutively secreted proteins did not bind. The receptor has been purified, cloned and antisense studies indicate that it functions in vivo to sort prohormones to the regulated secretory granules for processing and secretion. The novel class of prohormone converting aspartic proteases was studied. One member of this class, yeast aspartic protease 3 (YAP3p) has been shown to cleave at mono- and paired basic residue sites of mammalian prohormones. Specificity studies on YAP3p showed an enhancement in efficiency of cleavage of prohormones having additional basic residues upstream and/or downstream of the cleavage site. Substrates with an Arg basic residue in the P2' position was the most efficiently cleaved. Modeling studies revealed an acidic residue, Asp in the S2' pocket which may explain this increased efficiency. Biosynthesis studies showed that YAP3p is synthesized as a pro-enzyme which is autocatalytically cleaved to form the active enzyme. It can be further cleaved at an Asn residue to yield alpha and beta subunits which are then linked by a disulfide bridge to form a heterodimer. Antibodies against YAP3p were shown to cross-react with the purified, bovine intermediate pituitary POMC converting enzyme (PCE) an 70 kD basic residue-specific aspartic protease, indicating homology between them. In addition, a ~90 kDa anti-YAP3p immunoreactive protein was found in hypothalamus and anterior pituitary secretory granules. The ~90 kD YAP3-related protein was distributed in peptide-rich brain regions and co-localized with CCK, and vasopressin mRNA in rat brain neurons. Thus, YAP3p-related aspartic proteases, including PCE, likely play an important role in prohormone processing in mammalian brain. Several clones have now been obtained using the YAP3p antibody to screen a mouse brain expression library and they are currently being sequenced.

Project Description

Objectives:

The ACTH-endorphin family of peptides play many physiological roles (e.g. in stress, fetal development, osmoregulation and adrenal regeneration). These peptides are derived from a single multivalent prohormone (pro-opiomelanocortin, POMC). This prohormone is proteolytically processed at paired and tetrabasic residue sites to yield various active peptide products (α-MSH, ACTH, β-endorphin, β-LPH, N-POMC$_{1\text{-}49}$, γ_3-MSH) which, in turn, are modified at the C- and/or N-terminus. Differential processing occurs in different brain and pituitary regions. Proteolytic processing of POMC begins in the trans-Golgi network but occurs primarily within the secretory granule (vesicle). A highly specific set of proteases are involved in the various cleavages of this prohormone. The prohormone is differentially processed in various brain and pituitary regions, giving rise to a large diversity of secreted end-products with different biological actions. Moreover, the processing also appears to be modifiable by physiological inputs to the tissue. Our long-term goal is to understand the molecular mechanisms involved in the intracellular trafficking and sorting of POMC to the secretory granule for processing and the regulation of tissue- and stimulus-specific processing of this prohormone by different processing enzymes. Such cellular mechanisms uncovered for POMC will likely be extendable to other prohormones and pro-neuropeptides. Elucidating the molecular mechanisms which regulate the intracellular trafficking, biosynthesis and secretion of the POMC-derived peptides will facilitate the overall understanding of the regulation of the hypothalamic-pituitary-adrenal (HPA) axis in normal and disease states. We have chosen yeast cells, the frog and mouse brain, anterior and intermediate pituitary and bovine pituitary secretory vesicles as models and biological preparations, which are especially useful for addressing these issues. Our immediate major objectives are (i) to investigate the signals and mechanisms involved in the intracellular targeting and sorting of POMC into the regulated secretory pathway of endocrine and neuronal cells; (ii) to identify, purify, characterize and clone the enzymes involved in the processing of pro-opiomelanocortin to their respective hormones and to determine how these enzymes regulate tissue-specific processing; (iii) to study the regulation of biosynthesis (at the transcriptional, translational and post-translational levels), turnover and secretion of peptides that regulate the HPA axis such as ACTH, α-MSH, endorphin, vasopressin and pro-TRH-derived peptides, in the pituitary and CNS, during various types of pertubations (e.g. stress, salt-loading) and clinical paradigms.

Methods Employed

Acid-urea and SDS gel electrophoresis, high performance, and fast performance liquid chromatography (HPLC, FPLC), radioimmunoassays (RIA), immunochemistry, enzyme assays, column chromatography, antibody production, gene cloning techniques, *in situ* hybridization, electron microscopy and tissue culture.

Major Findings

Mechanism of sorting prohormones to regulated secretory granules

The search for the mechanism for sorting proteins including prohormones and pro-neuropeptides to the secretory granules of the regulated secretory pathway for processing and/or secretion in endocrine/neuroendocrine cells has been a major challenge in cell biology. Drs. David Cool, Emmanuel Norman, Fu-Sheng Shen and Winnie Tam have made great progress in elucidating the mechanism by which the prohormone, pro-opiomelanocortin (POMC) is sorted to the regulated secretory granules. We proposed the hypothesis that POMC and other prohormones are sorted to the regulated secretory pathway by a receptor-mediated mechanism, analogous to the sorting of lysosomal enzymes from the trans Golgi network (TGN) to the lysosome using the mannose-6-PO_4 receptor. In this model, POMC would bind *via* a sorting signal on the prohormone to membrane-bound receptors at the TGN. Subsequently, immature secretory granules would bud from the TGN delivering the prohormones into these organelles for cleavage by specific processing enzymes to active hormones. We have now succeeded in identifying consensus sorting signals in two prohormones, POMC and pro-enkephalin, and a sorting receptor which specifically binds the POMC sorting signal, pro-insulin, as well as other regulated secretory proteins.

The POMC sorting signal was identified using a series of deletion and substitution POMC cDNA mutants transfected into Neuro2a cells, a mouse neuroendocrine cell line. Transfected cells were assayed by immunocytochemistry and for stimulated secretion. POMC has a hairpin loop within the N-terminus 1-26 residues, which is stabilized by two disulfide bridges, Cys_2-Cys_{24} and Cys_8-$Cys_{20.}$ POMC mutants with the N-terminal hairpin loop residues 2-26 deleted; the Cys_8-Cys_{20} disulfide bridge deleted; the two disulfide bridges Cys_8-Cys_{20} and Cys_2-Cys_{24} deleted; or with the residues Cys_8 to Cys_{20} deleted, were misrouted to the constitutive pathway. In contrast, wild type POMC and the mutant which contained one disulfide bridge Cys_8-Cys_{20} intact, was correctly sorted to the granules of the regulated secretory pathway. This data showed that a motif containing residues 8-20 and one disulfide bridge (Cys_8-Cys_{20}) was not only sufficient, but necessary for the sorting of POMC to the regulated secretory pathway. In collaboration with Dr. Christopher Snell (Sandoz Institute, London), this hairpin loop region of N-POMC (Cys_8 to Cys_{20}) was modeled and identified as an amphipathic loop with two lobes containing four highly conserved amino acid residues (Asp_{10} Leu_{11} Glu_{14} Leu_{18}) which are exposed on the surface of the loop. This 13 amino acid amphipathic loop structure is stabilized by the disulfide bridge, Cys_8-Cys_{20}. This model has been confirmed by NMR studies with the N-$POMC_{1-26}$ peptide, done in collaboration with Dr. Andrew Bird (NCI) and Dr. Lewis Parnell (NIDDK).

Recently, Dr. Shen and Winnie Tam have analyzed the effect of substituting the four highly conserved residues at the surface of the loop to Ala_{10} Ser_{11} Ala_{14} Ser_{18}, on routing of POMC. This mutant POMC was transfected into Neuro2a cells and found not to be routed to the regulated secretory pathway, showing the physiological importance of these four residues (D_{10} L_{11} E_{14} L_{18}), perhaps in binding the sorting receptor.

The sorting signal of another prohormone, pro-enkephalin was also identified. Based on molecular modeling studies using the POMC sorting signal as a template, carried out by Dr. Snell, the N-terminal 31 residues of pro-enkephalin (N-Pro-enk_{1-31}) was found to have a sorting signal motif similar to that of POMC. N-Pro-enk_{1-31} contains a 19 amino acid amphipathic loop and two disulfide bridges, Cys_2-Cys_{28} and Cys_6-Cys_{24} determined experimentally by mass spectroscopy done in collaboration with Dr. Parnell (NIDDK). N-Pro-enk$_{1-31}$ when fused to the bacterial protein, chloramphenicol acetyltransferase (CAT) was shown to direct CAT to the regulated secretory pathway. Removal of the disulfide bridge between Cys$_6$-Cys$_{24}$ by mutation of Cys$_{24}$ to Ser$_{24}$ resulted in the mistargeting of this mutant to the constitutive pathway. Thus, we have identified the amphipathic loop structure consisting of N-Pro-enk$_{1-24}$, stabilized by one disulfide bridge, Cys$_6$-Cys$_{24}$, as a consensus, conformation-dependent sorting signal motif for directing pro-enkephalin to the regulated secretory pathway .

Dr. Cool has characterized a sorting receptor for the regulated secretory pathway using iodinated N-POMC$_{1-26}$, the sorting signal, as ligand. This ligand was shown to bind to the luminal side of purified secretory granule membranes from bovine intermediate pituitary with low affinity (K_d=6 μM) and a maximal binding capacity (B_{max}) of 580 pmoles/mg protein. Binding of N-POMC$_{1-26}$ was pH-dependent with the optimum pH being 5.5-6.0. The binding was highly specific for N-POMC$_{1-26}$, with no binding demonstrated for the mid-portion and C-terminal fragments of POMC, or constitutively secreted proteins including immunoglobulin G and pro-albumin. Full-length POMC, pro-insulin, pro-enkephalin and chromogranin A bound to these membranes in an N-POMC$_{1-26}$ displaceable manner. Moreover, pro-insulin was shown to bind the secretory granule membranes with a similar Kd as POMC. Similar binding occurred for Golgi but not plasma membranes. Crosslinking of the ^{125}[I]-N-POMC$_{1-26}$ to the secretory granule membranes, followed by SDS-PAGE revealed that ^{125}[I]-N-POMC$_{1-26}$ bound to a 54 Kd protein and that this binding was displaced by unlabeled N-POMC$_{1-26}$. This sorting receptor protein was purified to homogeneity and the N-terminal amino acid sequence determined by Dr. H-C Chen (ERRB). Dr. Emmanuel Normant has recently cloned the protein and constructed an antisense mRNA for knock out experiments. Antibodies have also been generated to the protein. Preliminary studies by Drs. Cool and Normant indicate that in Neuro2a cells that show >90% antisense knock out of expression of the sorting receptor, transfected POMC was not targeted to the regulated secretory pathway but was secreted constitutively. These studies provide physiological evidence that the N-POMC$_{1-26}$ binding protein we isolated was indeed a sorting receptor for prohormones and perhaps also for other proteins destined for the regulated secretory pathway. Furthermore, Dr. Shen is currently analyzing a mutant mouse with the sorting receptor knocked out, to determine the ability of POMC to be targeted to secretory granules in the pituitary of these animals. Thus, our studies have identified the consensus sorting signals on prohormones and a sorting receptor for these signals, providing strong evidence in support of a receptor-mediated mechanism for sorting proteins to the regulated secretory pathway.

Novel prohormone converting aspartic proteases

Peptide hormones and neuropeptides are synthesized as larger polypeptides that undergo a series of post-translational modifications to produce bioactive peptides. Important among these modifications is the limited proteolytic cleavage step performed by endopeptidases at specific paired basic residue sites of the precursors. Two major classes of endopeptidases, serine and aspartic proteases appear to be involved in this type of processing. We have focussed on the characterization of the aspartic protease class of proprotein converting enzymes. Several members of this class have been identified, including yeast aspartic protease 3 (YAP3p/YAPsin 1), yeast Mkc 7 (YAPsin 2), and mammalian pro-opiomelanocortin converting enzyme (PCE/YAPsin 3). YAP3p and Mkc 7 are highly homologous based on their primary sequence and an antibody against YAP3p showed cross-reactivity with PCE, indicating that these three enzymes are structurally related. Moreover, these aspartic proteases have unique specificity for mono as well as paired basic residue sites, unlike other aspartic proteases. These three enzymes have now been classified as YAPsins since they also share homology with pepsin.

Dr. Niamh Cawley and Vicki Olsen have continued to study the biosynthesis and specificity of YAP3p as a model enzyme of the YAPsins. YAP3p was shown to be synthesized as a pro-enzyme which was subsequently cleaved on the carboxyl side of the Lys_{66}-Arg_{67} pair to remove the pro-region, yielding an active enzyme. Activation occurred at an acidic pH (4.5) and was autocatalytic, as evidenced by the lack of removal of the pro-region in an enzyme which had the active site Asp mutated. Subsequent to activation, YAP3p was further cleaved between Asn_{144}-Asp_{145} to yield an α and a β subunit. These two subunits were linked together by a disulfide bridge to form an enzymatically active heterodimer.

YAP3p is a membrane-associated enzyme which is attached to the membrane *via* a glycosyl phosphatidyl inositol (GPI) anchoring site at the C-terminus of the protein. Vicki Olsen showed that using different deletion mutants the last 17 hydrophobic amino acid residues at the C-terminus were essential for GPI binding.

The kinetics of processing of various prohormones at paired basic residues by YAP3p was studied by Dr. Cawley. He showed that additional basic residues upstream and downstream of the cleavage site of the substrate increased the efficiency of cleavage. Unlike the prohormone convertases (PCs) of the serine protease class, YAP3p cleaved pro-proteins at mono as well as paired basic residue sites. Recently, Vicki Olsen examined the effect of basic residues up- and downstream of a single lysine processing site in CCK_{13-33}, on the efficiency of cleavage by YAP3p. CCK_{13-33} contains an Arg in the P6 position (upstream) relative to the cleavage site Lys (P1 position). Removal of this upstream Arg at the P6 position was found not to impair cleavage at the monobasic cleavage site, however, the relative efficiency (Kcat/Km) decreased 2-fold compared to the normal CCK_{13-33} substrate. Introduction of additional arginine residues downstream at the P2' and the P6' positions increased the catalytic efficiency 19- and 7-fold respectively, relative to the wild-type substrate. This study revealed that an Arg in the P2' position greatly enhanced the cleavage efficiency. This finding was further substantiated using other substrates, pro-albumin peptides and adrenomedullin done in collaboration with Drs. Ledgerwood and Brennan. In collaboration with Dr. Tom Blundell, YAP3p has been modeled and shown to have an Asp^{291} at the S2' active site pocket. This provides an explanation for the enhanced efficiency of cleavage of the substrates with a P2' Arg, since the Asp^{291} charged residue would attract the P2' Arg. Consistent with this interpretation is the finding that when the P2' Arg was substituted for an Asp which can repel the Asp charge in the S2' pocket, the efficiency of cleavage fell to 40% of wild-type CCK_{13-33} and was 47-fold less than the CCK_{13-33} substrate with an Arg at P2'. Large scale production and purification of recombinant C-terminally truncated YAP3p is ongoing and near completion for crystallization and X-ray diffraction studies to be done in collaboration with Dr. Tom Blundell. Solving the crystal structure of YAP3p will confirm the computer model and provide a greater understanding of the structure/function and specificity of YAP3p.

We have recently identified pro-opiomelanocortin converting enzyme (PCE) previously purified in our laboratory as the first mammalian member of the YAPsin family. PCE can process prohormones including POMC, provasopressin and CCK at specific basic residue cleavage sites. Dr. Cawley showed that an antibody against YAP3p cross-reacted with purified bovine PCE on Western blot, indicating structural homology between these two enzymes, but not with other aspartic proteases such as renin or cathepsin D. A PCE-sized ~70 kD anti-YAP3p immunoreactive band was detected on Western blots of bovine intermediate and neural lobe secretory granules where PCE activity has been found. YAP3p antiserum also cross-reacted with a protein of ~90 kD from mouse hypothalamus and anterior pituitary, and bovine anterior pituitary secretory granules. This finding indicates that there is more than one mammalian homologue of YAP3p besides PCE. Distribution studies by Dr. L-P Pu showed the presence of anti-YAP3p immunopositive cells in bovine pituitary and peptide-rich brain regions, including the mouse arcuate nucleus and hippocampus, and the rat supraoptic nucleus (SON), paraventricular nucleus, cortex, striatum and reticular nucleus. In the bovine intermediate pituitary, a subpopulation of cells was intensely stained with the YAP3p antiserum, and in combination with *in situ* hybridization, these cells were shown to contain POMC mRNA. Only a subpopulation of cells was immunopositive for anti-YAP3p in bovine anterior pituitary and most of these cells were identified by double immunostaining with ACTH antiserum as corticotrophs. This suggests that YAP3-like enzymes play a role in POMC processing. *In situ* hybridization in combination with immunocytochemistry provided evidence for the localization of AVP mRNA in YAP3p immunopositive neurons in the rat SON, while CCK mRNA was detected in YAP3p immunopositive cells in the rat cortex and hippocampus. These results support a role of YAP3p-like aspartic proteases, including PCE in prohormone processing in endocrine/neuroendocrine cells *in vivo*.

Dr. Chun-Fa Zhang, Dr. Niamh Cawley, Ms. Ying Zhang and Ms. Vicki Olsen are currently attempting to clone mammalian homologues of the YAPsin family using several approaches including PCR with consensus aspartic proteases and YAP3 specific primers, as well as antibody screening of a mouse brain expression library. Two specific clones have been obtained using the antibody to

YAP3p. These clones are currently being sequenced to determine if they code for new and novel mammalian YAP3-related aspartic proteases, including PCE.

Significance of Biomedical Research and the Program of the Institute

The pro-opiomelanocortin family of peptides (ACTH, α-MSH and β-endorphin) which are synthesized both in brain and pituitary, have been implicated in higher brain functions (e.g., avoidance learning, analgesia and stress response) and the regulation of the hypothalamo-pituitary-adrenal axis. In addition α-MSH has been shown to have a role in neural regeneration, neuronal differentiation and osmoregulation. Studies on the regulation of the biosynthesis, intracellular transport post-translational modification and secretion of these pro-opiomelanocortin-related peptides in the nervous system of adult and developing animal models will ultimately provide insights into the integrative and regulatory roles these peptidergic neurons may play during development, as well as genetic diseases associated with defects in these cellular processes.

Proposed Course

In the coming year, Dr. David Cool will study the biosynthesis, intracellular trafficking and recycling of the sorting receptor. Dr. Fu-Sheng Shen will continue with the analysis of the mutant mouse lacking the sorting receptor. He will also study the mechanism of membrane anchoring of the sorting receptor using artificial bilayers, in collaboration with Dr. Joshua Zimmerberg's group. Winnie Tam will continue to study the role of the four highly conserved amino acids (D_{10} L_{11} E_{14} L_{18}) in the POMC sorting signal motif in binding to the sorting receptor. Dr. Emmanuel Normant will characterize the pH and ion dependency for aggregation of prohormones at the trans Golgi network. He will determine the role of this aggregation step in enhancing the efficiency of sorting of prohormones to the regulated secretory pathway.

Dr. Niamh Cawley together with Dr. Chun-fa Zhang, Ying Zhang and Vicki Olsen will characterize the YAP3p-related mammalian clones obtained from screening a mouse brain expression library using the antibody against the YAP3p processing enzyme. If a unique aspartic protease is cloned, it will be expressed and the enzyme activity and brain distribution studied. Vicki Olsen will also study the *in vivo* biosynthesis and processing of YAP3p using various yeast genetic mutants that are defective in certain intracellular transport steps.

Publications

Bamberger AM, Bamberger CM, Puy LA, Asa SL. Regulation of the GH gene family in placenta and pituitary. In: De Bellis A and Schipani E, eds. Future trends in endocrinology, Serono Symposia Series, Frontiers in Endocrinology 1995;Vol 14, pp 77-82.

Bamberger AM, Pu L-P, Cool DR, Loh YP. The neuro-2A cell line expresses [met]-enkephalin and vasopressin mRNA and peptide. Mol and Cell Endocrinol 1995;113:155-63.

Cawley NX, Pu L-P, Loh YP. Immunological identification and localization of yeast aspartic protease 3-like prohormone processing enzymes in mammalian brain and pituitary. Endocrinology in press.

Cool DR, Louie DY, Loh YP. Yeast aspartic protease 3 (YAP3P) is sorted to secretory granules and activated to process pro-opiomelanocortin in PC12 cells. Endocrinology (in press).

Friedman TC, Cool DR, Jayasvasti V, Louie D, Loh YP. Processing of pro-opiomelanocortin in GH3 cells: Inhibition by prohormone convertase 2 (PC2) antisense mRNA. Mol and Cell Endocrinol 1996;116:89-96.

Friedman TC, Loh YP, Cawley NX, Birch NP, Huang SS, Jackson IMD, Nillni EA. Processing of pro-TRH by bovine intermediate lobe secretory vesicle membrane PC1 and PC2 enzymes. Endocrinology 1995;136:4462-72.

Friedman TC, Mastorakos G, Bouzas EA, Newman TD, Mullen NM, Horton EG, Costello R, Papadopoulos NM and Chrousos GP. Carbohydrate and lipid metabolism in endogenous hypercortisolism: Shared features and metabolic syndrome X and NIDDM. Endocrine J (in press).

Friedman TC, Yanovski JA. Morning plasma free cortisol: Inability to distinguish patients with mild Cushing syndrome from patients with pseudo-cushing states. J Endocrinol Invest 1995;18:696-701.

Friedman TC, Yanovski JA, Nieman LK, Doppman JL, Cutler Jr., GB, Oldfield EH, Chrousos GP, Kalogeras KT. Inferior petrosal sinus arginine vasopressin concentrations in normal volunteers and patients with Cushing disease. J Clin Endocrinol Metab (in press).

Hill RM, Ledgerwood EC, Brennan SO, Pu L-P, Loh YP, Christie DL, Birch NP. Comparison of the molecular forms of the Kex2/subtilisin-like serine proteases SPC2, SPC3 and furin in neuroendocrine secretory vesicles reveals differences in carboxyl terminus truncation and membrane association. J Neurochem 1995;65:2318-26.

Ledgerwood EC, Brennan SO, Cawley NX, Loh YP, George PM. Yeast aspartic protease 3 (YAP3) prefers substrates with basic residues in the P_2, P_1 and P_2' positions. FEBS Lett 1996;383:67-71.

Loh YP, Snell CR, Cool DR. Evidence for a receptor-mediated mechanism for sorting pro-opiomelanocortin to the regulated secretory pathway. In: Krisch, B, ed. Advances in Life Sciences; Birkhäuser Press, Switzerland (in press).

Nillni EA, Friedman TC, Todd RB, Birch NP, Loh YP, Jackson IMD. ProTRH processing by PC1. J Neurochem 1995;65:2462-72.

Pu L-P, Ma W, Barker JL, Loh YP. Differential co-expression of genes encoding pro-TRH and prohormone convertases (PC1 and PC2) in rat brain neurons: Implications for differential processing of pro-TRH. Endocrinology 1996;137:1233-41.

Pu L-P, Van Leeuwen FW, Tracer HL, Sonnemans MAF, Loh YP. Localization of Vasopressin mRNA and immunoreactivity in pituicytes of pituitary stalk-transected rats after osmotic stimulation. Proc Natl Acad Sci USA 1995;92:10653-57.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00064-20 LDN

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Neurobiologic Studies of Neurons and Glia in Cell Culture

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.G. Nelson	Chief	LDN, NICHD
Others:	R. Davenport	NRC Fellow	LDN, NICHD
	M-X Li	Snr NRC Fellow	LDN, NICHD
	M. Jia	Visiting Associate	LDN, NICHD
	S. Kim	NRC	LDN, NICHD
	P. Latham	IPA	LDN, NICHD
	A. Parfitt	Expert	LDN, NICHD
	V. Dunlap	Bio Lab Tech	LDN, NICHD
	E. Thies	Technician	LDN, NICHD
	T. Le	Tech IRTA	LDN, NICHD

COOPERATING UNITS *(if any)*

Barry Richmond, Lab. of Neuropsychology, NIMH; Michael O'Donovan, Lab. of Neural Control, NINDS

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.8	5.8	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

a) A tissue culture chamber system allows independent electrical activation of each of two sets of cholinergic axons innervating a common population of skeletal muscle cells. Axonal activation produces a loss of synaptic inputs to the muscle as in the case during development *in vivo*. We have shown previously that the serine protease thrombin plays a critical role in this process of activity dependent synapse reduction or ADSR. We now show that activation of the thrombin receptor by a thrombin receptor activating peptide (TRAP) can produce synapse loss. An analysis of a TRAP analogue, which in other systems is inactive, also fails to produce synapse loss. TRAP action involves activation of a protein kinase and we find that staurosporine, a protein kinase blocker, blocks both TRAP elicited and electrically induced synapse loss. b) Theoretical models of neural networks have suggested that spontaneously active "pacemaker" neurons could be important determinants of network behavior. We have found (using both calcium imaging and patch electrode recordings) that in cultures in which all excitatory synaptic activity is blocked, some 10% or so of neurons exhibit "pacemaker" activity. c) Time lapse microscopy has defined three types of axonal responses to target cells and three categories of guidance molecules are postulated which are differentially distributed amongst tectal (chick) and superior colliculus (mouse) target cells. Neurons in both phyla have repellent cues localized in one particular target region, whereas glia from chick and mouse appear to express qualitatively different classes of guidance cues.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives

We continue our interest in the role of electrical activity in regulating development of the nervous system, in particular its synaptic circuitry. We are focusing on the role of proteases in the process of activity dependent synapse reduction (ADSR) and aim to elucidate the cell biologic mechanisms by which proteases act. We are also studying those molecular and cellular cues which are involved in neuronal interactions involved in synaptogenesis which may be independent of electrical activity. The relationship between these classes of phenomena should be important and we are using vertebrate cell culture systems to allow cellular and molecular understanding of them.

Methods Employed

A variety of dissociated cell culture preparations from the mammalian central nervous system are utilized, with emphasis on the fetal mouse spinal cord and skeletal muscle culture system. Gradient separation of large spinal cord neurons has allowed partial purification of motoneurons and improved the neuromuscular synaptic system. The electrophysiological techniques utilized include intracellular and patch electrode recording and voltage-clamping. Pharmacological agents are applied by local perfusion or bath application.

Calcium imaging was performed with calcium-sensitive dyes and a video/computer analytic system.

A culture system is being utilized which incorporates three chambers separated from one another by barriers which allow the growth of axons between chambers, but retain neuronal cell bodies within the chambers in which they are plated. Various markers or stains can be used to demonstrate axons that project from one chamber to the other, and the development of synaptic interactions between specific cell types can be followed morphologically and physiologically.

To assess developmental guidance mechanisms in the optic pathway of embryonic chick and mammals, we have dissociated retinal and tectal cells from animals at embryonic ages (E6-11 in chick; E13-16 in mouse) when retinal axons are just innervating their target nuclei *in vivo*. Target cells are dispersed usually in the center compartment and retinal cells or explant strips are placed in the side compartments of a redesigned Campenot chamber. We have replaced the teflon chamber barriers which separate compartments with glass coverslips to reduce the distance retinal axons must extend to reach the center compartment by approximately 10-fold. Cells are kept in culture for 2-10 days and assessed with time-lapse video microscopy or whole cell patch pipette recording.

Semiquantitative and quantitative polymerase chain reaction technology is used to measure a variety of mRNAs of interest.

Embryonic chick eggs are cracked on their 3rd day of incubation and cultured in a container designed after those used by Steve McLoon and colleagues (Univ of Minn). Until their 17th day overall of incubation, cultured chicks are maintained in controlled environment in the presence or absence of systemically applied protease inhibitors. Retrograde neuronal tracers are injected on the 16th day of incubation and retinae are subsequently removed and assessed for the number of ipsilaterally projecting retinal ganglion cells.

Major Findings

Mechanism of activity dependent synapse reduction

Previously we have shown that activity of the serine protease, thrombin, is essential for activity dependent synapse reduction (ADSR) at the rodent neuromuscular junction in our 3-compartment tissue culture system. Thrombin has two modes of action, the first being that of a classical protease as exemplified by its cleavage of fibrinogen to produce fibrin, the end product of the coagulation cascade. Secondly, thrombin has been shown to cleave an integral membrane protein, the thrombin receptor, to produce a new amino terminus peptide which acts as a "tethered ligand" to activate the receptor and produce a number of cellular responses (including astrocyte shape changes and inhibition of neural growth cone activity). Synthetic peptides are available with the same amino acid sequence as the revealed 'tethered ligand' portion of the thrombin receptor. These peptides activate the thrombin receptor and are called Thrombin Receptor Activating Peptides or TRAPs and mimic the action of thrombin in several preparations. An attractive feature of this system is that peptides with the same amino acid composition as the TRAP are available which have the first two amino acids reversed and are inactive. Thus, SFLL is an active sequence while FSLL is completely inactive. We have applied these peptides to our synaptic preparation and find that the SFLL sequence produces a dose- and time-dependent loss of synapses, while the FSLL peptide produces no such loss. In other preparations the effect of thrombin receptor activation has been shown to be blocked by inhibitors of protein kinases such as staurosporine. We find that coapplication of the SFLL and staurosporine does not result in synapse loss; staurosporine blocks the action of the TRAP. Furthermore, the synapse loss due to electrical activation (The ADSR) is also blocked by 3 nM staurosporine. Preliminary results indicate that H-7, another protein kinase blocker, blocks TRAP action, but calphostin does not. These results strongly suggest that the ADSR involves thrombin activation (via the thrombin receptor) of a protein kinase, although the limited specificity of the blockers does not allow definitive identification of which protein kinase may be involved.

Glia cells are responsible for cholinergic deficit in Down syndrome model.

The mouse model for Down syndrome (and indirectly for Alzheimer disease) is the TS16 mouse, trisomic for chromosome 16, the homolog of chromosome 21 in humans. We have made cultures of spinal cord neurons and cerebral cortical astroglia from trisomic animals and their euploid litter mates. These dissociated neuronal and glial preparations were put together in various combinations to determine whether the trisomic genotypes were associated with neuronal deficits and, if so, whether the neuronal or glial component of the cultures were responsible. The neuronal phenotype we have focussed on is cholinergic expression, since this is prominently depressed in both Down and Alzheimer patients. We found that a) cultures consisting of both TS16 neurons and TS16 glia had lower than control levels of choline acetyltransferase (ChAT) and fewer than normal numbers of ChAT-containing neurons. Total neuronal numbers in the TS16 cultures were not depressed from normal. When neurons from euploid animals were cultured with glia from TS16 animals, the ChAT levels of the cultures were depressed from normal. Furthermore, when TS16 neurons were grown on normal glia, some 'rescue' of cholinergic function was observed. Statistical analysis of the results indicated that the cholinergic phenotype was determined by the glial component of the cultures, not by the neuronal component. We hypothesize that excessive production of the β–amyloid precursor protein (the gene for which is located on chromosome 16 in the mouse) by TS16 glia may account for these findings.

Pacemaker cells drive activity in neural circuits

Neural networks generally exhibit rather low rates of neuronal firing in the absence of external inputs. The basis for this is unclear and we have developed analytic models which generate firing patterns and response to inputs with which hypotheses as to mechanisms responsible for network behavior can be tested. Experimental data are obtained from cultures of spinal neurons with sensory connections in our 3-chamber culture system. One of the predictions of our models is that the cultures should contain cells that fire spontaneously ('pacemaker cells") even when all excitatory synaptic activity is blocked. Recently we have verified the existence of pacemaker cells that fire regularly at 5-15 Hz. Preliminary results indicate that these cells comprise between 3% and 15% of the neurons in the cultures.

Axon Guidance Cues

Activity-independent cues are presumed to serve a role in the earliest mechanisms which lead to stereotyped neuronal circuit formation during development. We are examining activity-independent guidance components in the establishment of the retinotectal and retinocollicular systems of chick, mouse and rat. We established 3 categories for retinal ganglion cell growth cone behavior, as assessed during time lapse observations of their encounters with individual dissociated target cells. These have led to 3 categories of guidance components which are differentially distributed amongst tectal (chick) and superior colliculus target cells (mouse). Neurons in both phyla have repellent cues localized to one particular target region, whereas glia from chick and mouse appear to express qualitatively different classes of guidance components.

A transient ipsilateral projection from retinae to optic tecta in the embryonic chick is subsequently lost through presumptive competitive interactions with contralateral projections. We are investigating the possible role of protease/protease inhibitors on this projection, based in part on our previous work establishing the role of these molecules on the competitive interaction at neuromuscular synapses. Whole chick embryos are cultured in the presence or absence of protease inhibitors directed at serine proteases, and in particular thrombin. We have examined the loss of the projection during development in control animals and compared the remaining projection in treated versus control animals. Currently, we have only weak evidence for the effectiveness of several protease inhibitors.

Significance to Biomedical Research and the Program of the Institute

Assembling appropriate and functionally effective neural circuits is a critical developmental process of fundamental importance to the successful adaptation of the organism. Understanding the mechanisms involved in the process is clearly important for neuroscience and the NICHD, which has a lead role in dealing with problems of mental retardation and more subtle aspects of inadequate cognitive development. The role of the environment in general and electrical activity in neurons in particular may play in optimizing nervous system development is a central issue in this area.

Proposed Course

We will continue to pursue the cellular and molecular mechanisms involved in the ADSR. Further work with kinase inhibitors should allow us to characterize the kinase that is responsible. The location of the thrombin, the thrombin receptor and the protease nexin (PN1) needs to be established in order to develop an adequate model of the ADSR. Anatomical studies are required to delineate the pre- and post-synaptic elements which are the targets for thrombin action. Experiments to determine the basis for Hebbian vs. non-Hebbian ADSR are needed. A direct comparison of SCG and VH neurons should be made in this regard. The possible role of spontaneous activity in nerve and muscle in determining the stimulus specificity of the loss should be investigated. If we demonstrate that presynaptic changes are involved in ADSR, then studies of retrograde messengers such as nitric oxide and arachidonic acid will be necessary. We will examine cerebral cortical cultures to see if the pacemaker cells we have found in spinal cord cultures also occur in the cortical cultures. Detailed computer implementation of theoretical models will be made to explore the implications of pacemaker cells in central neural circuits.

We will continue to prepare aneural mouse skeletal muscle cultures in order to quantitate mRNA and protein levels of thrombin and PN-1, with and without cholinergic stimulation. Present evidence that the application of acetylcholine to electrically blocked muscle culture resulted in a 2-fold increase in prothrombin mRNA has involved semi-quantitative mRNA measurement and has given no indication of protein levels. Competitive RT-PCR and Western blot or quantitative ELISA will be performed to measure mRNA and protein levels of the two compounds. We will also investigate the role of muscle innervation in regulating the thrombin/PN-1 expression by using cholinergic neuron-muscle co-cultures in the 3-compartment chambers. Quantitation of the prothrombin and PN-1 mRNAs in the innervated muscle cells followed by electrical stimulation will be determined.

The time course of development of the thrombin and PN-1 *in vivo* will be investigated, particularly with reference to the time of maximal synapse elimination. Mouse skeletal muscle tissue will be taken at various stages of fetal and postnatal development. Quantitative determination of the mRNA and protein levels of thrombin and PN-1 will be done. Our preliminary results indicate that expression of prothrombin and PN-1 mRNAs in the muscle are regulated during development.

We also detected expression of thrombin receptor mRNA in the mouse muscle tissue and cultured cells. The codistribution of prothrombin with thrombin receptor mRNA in the muscle suggests that locally synthesized thrombin could be acting in an autocrine manner to regulate specific developmental process such as synapse remodeling. It is uncertain whether thrombin activates thrombin receptors on the pre- or postsynaptic membrane during synapse elimination. The cellular localization and quantitation of both the mRNA and protein of thrombin receptor will be investigated to evaluate the interaction between thrombin and its substrate thrombin receptor during synapse elimination in the neuromuscular junction.

We will continue our investigation of activity-independent guidance cues which help establish the stereotyped neuronal circuitry of the visual system. We will compare the cellular localization of avian and rodent cues. These phyla differ in their early axonal projections from the retinae to their CNS targets, but are refined to appear similar by adulthood. Quantitative and qualitative differences in guidance components were detected and these may account for numerous differences during early development.

We will examine the ability of known guidance molecules constitutively expressed in carrier cells (NIH-3T3 cells) to elicit the particular growth cone behaviors that we have characterized. Also, we will examine the chick (tectal) and mouse (superior colliculus) target cells for the presence of particular guidance molecules by immunocytochemical and molecular biological techniques.

We will continue to characterize the ability of several serine protease inhibitors to affect the transient, ipsilateral retinotectal projection in embryonic chickens.

Protocols

Animals:

94-013	Nelson	Developmental neurobiology in culture systems

Publications

Davenport RW, Ping D, Mills LR, Kater SB. Distinct calcium signaling within neuronal growth cones and filopodia. J Neurobiol 1996;31:1-15.

Davenport RW, Thies E, Nelson PG. Cellular localization of guidance cues in the establishment of retinotectal topography. J Neurosci 1996;16:2074-85.

Festoff BW, Nelson PG, Brenneman DE. Prevention of activity-dependent neuronal death: vasoactive intestinal polypeptide stimulates astrocytes to secrete the thrombin-inhibiting neurotrophic serpin, protease Nexin I. J Neurobiol 1996;30:255-66.

Li MX, Jia M, Fields RD, Nelson PG. Modulation of calcium currents by electrical activity. J Neurophysiol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00094-25 LDN

PERIOD COVERED

October 1, 1995 - September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pineal Regulation: Environmental and Physiology Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and Institute affiliation)*

PI:	David C. Klein	Head	LDN, NICHD
Others:	Steven Coon	IRTA Fellow	LDN, NICHD

COOPERATING UNITS *(if any)*

Georgetown University (M.A.A. Namboodiri); University of Geneva (N. Schaad); University of Giessen (H. Korf)

LAB/BRANCH	Laboratory of Developmental Neurobiology
SECTION	Section on Neuroendocrinology
INSTITUTE AND LOCATION	NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF-YEARS: 1	PROFESSIONAL: 1	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

  ☐ (a1) Minors

  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project has been terminated.

PHS 6040 (Rev. (5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 00095-26LDN

PERIOD COVERED
October 1, 1995 - September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Pineal Regulation: Transsynaptic and intracellular mechanisms

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory and Institute affiliation)

PI:	D.C. Klein	Head	LDN, NICHD
Others:	J.L. Weller	Chemist	LDN, NICHD
	P. Roseboom	Staff Fellow	LDN, NICHD
	S. Coon	IRTA Fellow	LDN, NICHD
	R. Baler	Biotechnology Fellow	LDN, NICHD
	M. Bernard	Visiting Fellow	LDN, NICHD
	J. Gastel	IRTA Fellow	LDN, NICHD

COOPERATING UNITS (if any)
Georgetown Univ (M.A.A. Namboodiri); Frankfurt Univ (H. Korf); NIMH (M. Zatz, D. Jacobowitz, S. Young); NEI (I. Rodriguez); Emory Univ (M. Iuvone); Texas A&M (V. Cassone); U. Poitiers (P. Voison); Johns Hopkins Univ (S. McCune)

LAB/BRANCH Laboratory of Developmental Neurobiology

SECTION Neuroendocrinology

INSTITUTE AND LOCATION National Inst of Child Health and Human Development, NIH, Bethesda, MD 20892

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
9	7.9	1.1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Section on Neuroendocrinology conducts research on the pineal gland with heavy emphasis on the biochemistry and molecular biology of the two enzymes involved in the conversion of serotonin to melatonin. This indolic compound is synthesized at high levels at night in all vertebrates, due to a marked increase in the activity of the first enzyme in the serotonin melatonin pathway, serotonin N-acetyltransferase (arylalkylamine N-acetyltransferase, AANAT, EC2.3.1.87). The Section has determined the genetic code of the ovine, rat, human, and chicken forms of the enzyme. In all cases mRNA encoding the enzyme is ~1.0 to 1.5 kb. The human gene is ~2.5 kb. mRNA encoding this enzyme is found at high levels consistently in the night pineal gland; lower levels are found in the retina of most species. In addition, trace amounts are found in certain brain regions and in the pituitary gland. The levels of mRNA in the pineal gland exhibit a ~150-fold night/day rhythm in the rat, in which day values are nearly undetectable. The rhytym in the chicken is about 10- to 15-fold and that in the sheep is not more than 1.5 fold. Expression of the AA-NAT gene in the rat is regulated by a cyclic AMP mechanism, in which expression is turned on by phosphorylation of a cyclic AMP response element binding protein. This induces expression of AA-NAT and coinduces the expression of genes encoding two inhibitory transcription factors, at least one of which seems to play a role in rapidly turning off expression of the AA-NAT gene. Analysis of the protein encoded by the AA-NAT gene reveals that it is distantly related to a family of acetyltransferase molecules and that AA-NAT represents a new subfamily.

Project Description

Objectives:

To describe the transsynaptic and intracellular mechanisms through which neuronal activity regulates the biochemical activity of a neural tissue, the pineal gland.

Methods Employed:

General: Tissue is obtained from normal animals or surgically prepared animals. Cultures of pineal tissue are used to study biochemical activity under highly defined conditions. Pineal glands and dispersed suspension cultures of pineal cells are used. Tissues are treated with chemicals of interest and the activity of enzymes and concentrations of cell chemicals are measured by a variety of techniques. To study development, pineal glands are removed from rats at sequential stages of development and enzyme and metabolic chemicals are measured. Enzymes are purified by a variety of methods. Antisera are produced in rabbits; antisera are used to identify proteins. cDNA libraries are prepared using a λgt11 vector and screened with synthetic oligonucleotides and cDNA probes.

Pineal tissue was obtained from normal animals or surgically operated animals. Pineal enzymes are measured by radiochemical techniques. Proteins are resolved by polyacrylamide gel electrophoresis and electroblotted to membranes for immunological analysis. Total RNA is isolated by a single step method using acid guanidinium thiocyanate-phenol-chloroform extraction; resolved on an agarose gel and transferred onto positively charged membranes for message level analysis. In addition, polymerase chain reaction technology is used to confirm the results obtained by Northern analysis.

Transcription factors: Analysis is through use of highly specific antiserum, combined with labelled oligonucleotide probes in gel shift assays. In addition, PCR is used to detect mRNA transcripts as is Northern blot analysis.

Major findings:

Serotonin N-acetyltransferase (arylalkylamine N-acetyltransferae, AA-NAT, EC2.3.1.5): An expression cloning technique was used to initially identify a clone encoding serotonin N-acetyltransferase. This work was done by a team headed by Steven Coon. The identity of the clone was verified by Ruben Baler, who expressed it in several systems and by Joan Weller and Steve Coon who determined that the expressed protein had substrate specificity similar to that in partially purified preparations. This was extended by the cloning of cDNA encoding the human , rat and chicken forms of the enzyme. Tissue distribution studies indicate that there is little or no expression in any species in any endocrine or internal organ and that there is weak expression in the brain. Expression of a variable nature is detected in the retina, and high detection occurs in the pineal at night.

Analysis of the predicted proteins encoded by AA-NAT transcripts reveals the protein is organized into putative AcCoA binding sites, putative active site region, and putative arylalkylamine binding sites.

Rat AA-NAT mRNA: Studies by Patrick Roseboom have established that rat AA-NAT mRNA is strongly expressed in the pineal gland and to a lower degree in the retina. Transcripts can also be detected in the brain. Pineal expression increases >150-fold at night. This requires intact adrenergic innervation of the pineal gland and appears to be driven by the clock in the suprachiasmatic nucleus. Expression can be induced by treatment with adrenergic agents and cyclic AMP protagonists. Expression is enhanced by treatment with a protein synthesis inhibitor, indicating that a negatively acting transcription factor may be coinduced with AA-NAT. Jonathan Gastel and Joan Weller have developed and used a set of antisera to find that rapid changes in activity of the enzyme are accompanied by a rapid loss of immunodetectable protein, but not by a rapid decrease in AA-NAT mRNA. These studies established that AA-NAT activity is regulated in the rat at multiple levels involving both mRNA and protein.

Sheep AA-NAT mRNA: Studies by Steve Coon established that the amount of pineal AA-NAT mRNA does not change markedly over a 24 hour period. His studies have indicated that less than 50% of the changes in AA-NAT mRNA occur when activity changes over 15-fold. Studies by Joan Weller have revealed that immunoreactive AA-NAT protein levels generally follow levels of enzyme activity, suggesting that the increase in enzyme activity is due to a post transcriptional mechanism, because mRNA levels did not change. These studies provide clear evidence that in the sheep pineal gland AA-NAT activity is regulated primarily at a post transcriptional level.

Human AA-NAT mRNA: Studies done by Steve Coon and Ignacio Rodriguez (NEI) have resulted in the cloning of the human AA-NAT gene and the localization of the gene to chromosome 17q26. The gene is expressed at relatively high levels in most of the pineal glands and retinae examined. In addition, trace levels are also found in the brain. A single strand conformation polymorphism assay has been established to screen patients for polymorphism and blood has been collected from several subjects for analysis.

Chicken AA-NAT mRNA: Studies by Marianne Bernard have been done with collaborators at the NIMH and Emory University. These efforts have revealed that the distribution of AA-NAT mRNA is generally similar in the chicken as it is in mammals, with high levels occurring only in the pineal gland and retina. There is a ~10-fold rhythm in AA-NAT mRNA in both tissues and the rhythm in AA-NAT mRNA in the pineal gland persists in constant lighting and darkness. This indicates that the rhythm is generated by an endogenous clock. *In vitro* studies indicate that the rhythm also persists in cell culture. This means that the pineal clock, which has been well described, drives the rhythm in AA-NAT mRNA.

Light influences the rhythm in AA-NAT mRNA by causing a phase shift in the rhythm. Light does not, however, rapidly suppress AA-NAT mRNA, although it does cause a rapid decrease in AA-NAT activity. This indicates that the rhythm in AA-NAT activity reflects clock driven changes in the abundance of AA-NAT mRNA and that light can act on the clock to shift the phase of this rhythm. In addition light can act on this system at a post transcriptional level to regulate enzyme activity.

Studies on AA-NAT protein: AA-NAT protein is being studied using a number of antisera that are being developed primarily by Jonathan Gastel and Joan Weller. This effort has generated anti-peptide sera directed against highly conserved portions of the protein, including N- and C- terminal sequences which contain phosphorylation sites, highly conserved hydrophobic sites thought to bind arylalkylamines, and motif A, which is thought to be part of the AcCoA binding site. In addition, antisera is being raised against expressed protein. These sera are being used to study the regulation of AA-NAT protein in *in vivo* and *in vitro* studies. A goal of these studies is to develop antisera that will be useful for micro immunoprecipitation purification of the protein and for immunohistochemistry.

A second approach to the analysis of the AA-NAT protein is the preparation of highly purified protein for physical analysis by X-ray crystallography and NMR spectroscopy. The expression and purification work is being done by Jonathan Gastel. These approaches will identify specific binding sites and the catalytic site of the enzyme.

HIOMT: Efforts were made to detect HIOMT in the human retina by several techniques, including Northern blot, enzyme assay and Western blot. This project was led by Marianne Bernard. These methods did not detect HIOMT, although it was possible to detect low amounts of HIOMT mRNA using a highly sensitive PCR technique. These findings suggest that the retina may not synthesize significant amounts of melatonin.

Dr. Bernard studied the regulation of expression of the HIOMT gene in Y-79 cells and made two important discoveries. One is that HIOMT expression is strongly dependent upon a serum factor, and that removal of serum results in a rapid loss of enzyme activity and mRNA encoding HIOMT. The second finding of special importance is that HIOMT can be regulated by retinoic acid, suggesting that this might be involved in the regulation of HIOMT *in vivo* and the retinoic acid or a related compound might be the factor in serum which regulates HIOMT expression.

Transcription factors: Ruben Baler has lead an effort to identify a Fos-related protein in the pineal gland that is remarkably regulated. The abundance of this immunological signal increased 100-fold at night and is regulated by the same mechanism which controls expression of the AA-NAT gene. Baler discovered that this protein is a fos related protein, identified as Fos related antigen 2. He was the first to clone this in the rat and his work now is directed to determining how this transcription factor is involved in regulation of the expression of AA-NAT.

It appears that expression of the Fra-2 gene is turned on by norepinephrine acting through an adrenergic-cyclic AMP mechanism, and that this immediately results in a large increase in Fra-2 protein. This coinduction with AA-NAT represents a mechanism through which expression of the AA-NAT gene turns off the expression of both AA-NAT and Fra-2, so that the abundance of both messages and their corresponding proteins decrease to undetectable levels during the day.

The nuclear orphan receptor RZRβ was found by Ruben Baler and Steven Coon to be highly expressed in the rat pineal gland. Recent studies proposed that melatonin, the pineal hormone which regulates a wide variety of circadian-linked phenomena, may be the natural ligand of this receptor. These provocative reports prompted them to learn more about RZRβ and how it might function in circadian physiology. They determined that there was high expression of this receptor in the pineal gland, and that pineal RZRβ expression exhibits a strong circadian pattern. Expression is under photoneural regulation and involves an adrenergic cAMP mechanism.

Adrenergically induced proteins: The most prominent adrenergically-stimulated protein in norepinephrine-treated glands, as judged by incorporation of ^{35}S-methionine into proteins has a molecular weight of about 37 kDa and a pI of about 6. This protein was partially purified and resolved by two-dimensional gel electrophoresis. It was identified by microsequencing to be an isoform of malate dehydrogenase. This was confirmed by immunochemical techniques, using antisera raised against synthetic malate dehydrogenase peptides. It was also determined that the appearance of this protein reflects two processes, new synthesis of protein and cotranslational phosphorylation.

Significance to Biomedical Research and the Program of the Institute:

The pineal gland offers an attractive model system for the study of the neurochemical transduction from the receptor to the gene. This is a fundamental aspect of brain function and further understanding will lead to better treatment of neurological and mental diseases, and a better understanding of problems of mental retardation.

Proposed Course:

N-Acetyltransferase: The promoter will be identified and the factors regulating expression of this gene will be determined, including transcription factors and factors determining tissue specificity.

Factors regulating the activity of the enzyme will be analyzed using expression systems, with special attention paid to the role of phosphorylation of two putative protein kinase A sites. The mode of degradation of the protein will be determined.

Human subjects will be studied to determine whether there are AA-NAT polymorphisms and whether these are of functional importance.

Transcription factors: Studies on the promoter will focus on the relative importance and the interaction of positive and negative transcription factors. The primary positive transcription factor to be studied is CREB and the negative transcription factors will be Fra-2 and ICER.

Protocols:

Animal

93-005	Klein	Mouse urinary 6-sulfatoxy melatonin. . .
93-016	Klein	Neuroendocrine regulation: Rat pineal gland
95-027	Klein	Raise goat anti-serotonin N-acetyltransferase
95-028	Klein	Ovine serotonin N-acetyltransferase

Human

96-CH0019	Klein	Melatonin synthesis genes in subjects with atypical melatonin production

Publications:

Baler R, Coon SL, Klein DC. Orphan nuclear receptor RZRβ: Cyclic AMP regulates expression in the pineal gland, Biochem Biophys Res Commun 1996;220:975-8.

Baler R, Klein DC. Circadian expression of transcription factor Fra 2 in the rat pineal gland, J Biol Chem 1995;270:27319-25.

Baler R, Klein DC. Circadian regulation of transcription factor FRA-2 in the rat pineal gland. In: Advances in Pineal Res, 1996; Vol 9 (in press).

Bernard M, Donohue SJ, Klein DC. Human hydroxyindole-O-methyltransferase in pineal gland, retina and Y-79 retinoblastoma cells, Brain Res 1995;696:37-48.

Bernard M, Iuvone PM, Cassone VM, Roseboom PH, Coon SL, Klein DC. Chicken arylalkylamine-N-acetyltransferase: Cloning and circadian regulation in the pineal gland. In: Advances in Pineal Research, 1996; Vol 9 (in press).

Bernard M, Iuvone PM, Cassone VM, Roseboom PH, Coon SL, Klein DC Melatonin synthesis: Photic and circadian regulation of serotonin *N*-acetyltransferase mRNA in the chicken pineal gland and retina, J Neurochem (in press).

Bernard M, Klein DC. Melatonin synthetic enzyme: Retinoic acid increases hydroxyindole-O-methyltransferase activity and mRNA in human Y-79 retinoblastoma cells, J Neurochem (in press).

Bernard M, Voisin P, Klein DC. Hydroxyindole-O-methyltransferase in Y-79 cells: Regulation by serum, Brain Res 1996;727:118-24.

Coon SL, Mazuruk K, Bernard B, Roseboom PH, Klein DC, Rodriguez IR. The human serotonin *N*-acetyltransferase (EC 2.3.1.87) Gene: Structure, chromosomal localization and tissue expression, Genomics 1996;34:76-84.

Coon SL, Roseboom PH, Baler R, Weller JL, Namboodiri MAA, Koonin EV, Klein DC. Pineal serotonin N-acetyltransferase (EC 2.3.1.87): Expression cloning and molecular analysis, Science 1995;270:1681-83.

Klein, D.C., Coon, S.L., Krzysztof, M., Bernard M., Roseboom, P.H., and Rodriguez, I.R. (1996) Biological rhythms and psychiatric disease: Focus on melatonin-related genes. (in press).

Klein DC, Roseboom PH, Coon SL. New light is shining on the melatonin rhythm enzyme: The first post cloning view, Trends in Endocrinol Metabol 1996;7:106-12.

Klein DC, Coon SL, Mazuruk K, Bernard M, Roseboom PH, Rodriguez IR. Biological rhythms and psychiatric disease: Focus on melatonin-related genes. In: Wahlstrom J,Wetterberg L, eds. Genetics and Psychiatric Disorders. Oxford: Elsevier Science (in press).

Roseboom PH, Coon SL, Baler R, McCune SK, Weller JL, Klein DC. Melatonin synthesis: Regulation of the more than 150-fold nocturnal increase in serotonin N-acetyltransferase messenger RNA in the rat pineal gland. Endocrinology 1996;137:3033-44.

Weller JL, Jaffee H, Roseboom PH, Zylka M, Klein DC. 2D-PAGE analysis: Adrenergically regulated pineal protein AIP 37/6 is a phosphorylated isoform of cytosolic malate dehydrogenase, Brain Research 1996;713:8-16.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00704-12 LDN

PERIOD COVERED

October 1, 1995 - September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tetanus Toxin Effects and Localization in Neurons

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Elaine A. Neale	Head	LDN, NICHD
Others:	Lura C. Williamson	Sr. Staff Fellow	LDN, NICHD
	Linda M. Bowers	Biologist	LDN, NICHD
	Veronica Dunlap	Biol. Lab. Tech.	LDN, NICHD
	Adam J. Weber	Summer IRTA	LDN, NICHD
	Sara E. Behar	Summer IRTA	LDN, NICHD
	Violet Aldaia	Special volunteer	LDN, NICHD

COOPERATING UNITS (if any)

Div. Bacterial Products, Ctr for Biologics Evaluation and Research, Food and Drug Adm., J. H. Halpern, W.H. Habig); NIDR (S.H. Leppla); US Army Med. Res. Instit. of Infectious Diseases (J.E. Brown);Dipartimento di Science Biomed. Sperimentali, Univ. of Padova (C. Montecucco)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Cell Biology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.95	1.4	1.55

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Clostridial neurotoxins have profound effects on neuronal membrane traffic. Each of the toxins inhibits neurotransmission, cleaves a specific protein implicated in synaptic vesicle docking/fusion, and prevents the fusion of synaptic vesicles with the presynaptic membrane. Botulinum neurotoxin C is unique in that it proteolyzes two proteins (SNAP-25 and syntaxin) and is severely cytotoxic to developing and mature neurons in cell culture. Botulinum neurotoxin A uncouples vesicle endocytosis from exocytosis; it blocks synaptic vesicle exocytosis but allows stimulus-triggered synaptic vesicle membrane retrieval. Neuronal membrane gangliosides play an important role in tetanus toxin internalization and may be a functional component of the toxin receptor. Studies with chimeric toxins indicate that either the number or distribution of toxin receptors underlies the different effects of toxins on inhibitory and excitatory neurotransmitter release. Specific receptors likely account for the greater efficacy of botulinum neurotoxin A at nerve-muscle synapses and tetanus toxin at spinal cord synapses.

Project Description:

Objectives:

To analyze the presynaptic action of the clostridial neurotoxins at the biochemical and ultrastructural levels.

Methods Employed:

Primary neuronal cell culture; morphologic techniques including light, video, fluorescence, and electron microscopy, immunohistochemistry and immunoelectron microscopy; computer-assisted image analysis and morphometry; biochemical techniques including scintillation spectroscopy, thin-layer chromatography, and gel electrophoresis and immunoblot.

Major Findings:

Clostridial neurotoxins (CNTs) include tetanus toxin (TeNT) and the seven serotypes of botulinum neurotoxin (BoNT). The CNTs are zinc endopeptidases which block neurotransmission and have been shown to cleave in vitro specific proteins involved in synaptic vesicle docking and/or fusion. Tetanus toxin and BoNTs B, D, F and G cleave vesicle-associated membrane protein (VAMP), also known as synaptobrevin. Synaptosomal-associated protein of 25 kDa (SNAP-25) is cleaved by BoNTs A and E, and BoNT C (in vitro) cleaves syntaxin. We recently reported that, in intact neurons, BoNT C not only cleaves syntaxin, but acts also on SNAP-25 (Williamson et al., 1996). As such, BoNT C is the only CNT to act on two proteins implicated in neurotransmitter release. Furthermore, BoNT C is severely cytotoxic to both developing and mature neurons in cell culture, having no effect on the glial cells in these cultures. When BoNT C is added at the time of plating, the few neurons surviving after five days are stunted in terms of neurite outgrowth and synaptogenesis. BoNT A, which also cleaves SNAP-25, has no effect on neuronal survival or on either neurite outgrowth or synaptogenesis. This difference suggests that syntaxin alone or in combination with SNAP-25 is an essential component of membrane trafficking during development. Disruption of membrane traffic may likewise underlie the toxicity in mature neurons. Another possibility, however, is that BoNT C cleavage of syntaxin affects the function of synaptic ion channels. Voltage-gated N, P and Q type calcium channels are implicated in neurotransmitter release and are reported to bind to syntaxin. In fully established cultures, we find that synaptic terminals are the primary site of BoNT C-induced neuronal degeneration. If syntaxin were involved in maintaining voltage-gated calcium channels in an inactivated state, cleavage of syntaxin by BoNT C might cause a shift toward the chronic activation of these channels. In support of this hypothesis, we find a 28% increase in ^{45}Ca uptake during the first 6 hours of BoNT C exposure compared to control or BoNT A-treated cultures.

We have correlated further the synaptic blockade induced by either TeNT or BoNT A with immuno-histochemistry of their respective protein substrates, with fine structure of the synaptic release site, with activity-dependent uptake of the dye FM1-43, and with stimulated uptake of horseradish peroxidase (HRP) into newly recycled synaptic vesicles. Potassium-evoked release of both an inhibitory (glycine) and an excitatory (glutamate) neurotransmitter is blocked completely in toxin-treated cultures. As mentioned above, immunoreactivity for VAMP and for SNAP-25 is abolished in cultures exposed to TeNT or BoNT A, respectively. Approximately twice the number of synaptic vesicles are "docked" (located within 10 nm of the membrane) at the active zones of toxin-blocked cultures as are seen in either spontaneously active or KCl-stimulated control cultures, evidence for a block in synaptic vesicle fusion. Consistent with the absence of synaptic activity, TeNT- (and BoNT B-, C-, or D-) treated cultures show no activity-dependent uptake of FM1-43. In contrast, cultures treated with high concentrations of BoNT A continue to show FM1-43 loading, indicating the persistence of synaptic vesicle endocytosis in the absence of exocytosis. These findings are confirmed by KCl stimulation in the presence of calcium and HRP. After two minutes of stimulation, approximately 10% of synaptic vesicles in control cultures contain HRP. TeNT-blocked cultures show HRP in only an occasional synaptic vesicle, whereas approximately 6% of synaptic vesicles become labeled in BoNT A-blocked terminals. These labeled vesicles appear to form via conventional clathrin-coated intermediate structures. No synaptic vesicle labeling occurs in the absence of calcium. These results strongly suggest that in BoNT A-blocked synaptic terminals, potassium stimulation is able to trigger synaptic vesicle membrane retrieval in the absence of synaptic vesicle exocytosis. To confirm the block in exocytosis, control and BoNT A-blocked cultures are loaded with HRP and subsequently depolarized with potassium to force vesicle fusion and exocytosis of HRP. In control terminals, the percentage of labeled synaptic vesicles decreases from 10% to 2.5%. Omitting calcium from the stimulation medium prevents the loss of HRP; HRP label persists in about 11% of synaptic vesicles. In contrast, in BoNT A-blocked cultures, the percentage of labeled vesicles increases from 6% to 9%. In these terminals, HRP may have moved from an intermediate membrane compartment(s) into synaptic vesicles, but there is no evidence that HRP is released from vesicles by exocytosis. Furthermore, the retrieval process is limited. An experiment in which BoNT A blocked cultures are depolarized repeatedly, with HRP added only during the final stimulation interval, shows that after five rounds of stimulation, it is no longer possible to internalize HRP into synaptic vesicles. Thus, the HRP filling of vesicles does not appear to be due to an unconventional exocytic process that continues during the depolarization. These observations suggest that BoNT A may be used to uncouple the cycle of triggered exocytosis-endocytosis that occurs at the synaptic terminal, allowing discrete analysis of the endocytosis component. They further suggest that VAMP (or another as yet unidentified substrate of tetanus toxin) may participate in the process of synaptic vesicle membrane retrieval.

We have examined the role of gangliosides as functional receptors for TeNT. Di- and trisialogangliosides "fix" TeNT, but controversy remains as to the importance of gangliosides as functional toxin receptors. We study spinal cord cell cultures grown in the presence of fumonisin B1, a mycotoxin which prevents ganglioside synthesis by inhibiting the activity of ceramide synthase. Fumonisin appears to be cytotoxic to glial cells, enabling us to grow neurons in the relative absence of glia. We find that spinal cord neurons grown in fumonisin develop dendrites, axons and synapses as demonstrated by immunohistochemistry using antibodies against microtubule-associated protein, against phosphorylated neurofilaments, and against synaptophysin, respectively. The loss of gangliosides is assessed using Fragment B of cholera toxin which binds to monosialogangliosides, or Fragment C of TeNT which binds to di- and trisialogangliosides, each conjugated to horseradish peroxidase (HRP). Virtually no cholera toxin or TeNT binds to these cultures. We have examined the action of TeNT in fumonisin-treated cultures. In cultures exposed to TeNT, the evoked release of glycine and glutamate is blocked, there is no evidence for activity-dependent uptake of the dye FM1-43, and VAMP immunofluorescence is lost from synaptic terminals immunostained with antibodies against synapsin 1. Chronic fumonisin has no effect on neurotransmitter release, FM1-43 uptake, or VAMP immunoreactivity. In fumonisin-grown cultures exposed to TeNT, neurotransmitter release continues at 75% of the control level, uptake of FM1-43 is still evident, and VAMP immunostaining persists in the majority of synaptic terminals labeled with anti- synapsin 1. Thus, the loss of gangliosides appears to protect neurons from the actions of TeNT, providing strong evidence that gangliosides are functional components of the receptors for TeNT.

The internalization, translocation, and trafficking of the clostridial neurotoxins address several intriguing issues in cell biology. The CNTs bind and are internalized at the neuromuscular junction. Whereas the BoNTs act at that site to block acetylcholine release causing the flaccid paralysis of clinical botulism, TeNT is routed to the spinal cord by retrograde transport in motor axons. This toxin crosses from the motor neuron into inhibitory interneurons where it blocks neurotransmitter release. The resulting disinhibition causes the spastic paralysis of clinical tetanus. We have begun to examine the action of the CNTs at the neuromuscular junction in multicompartment ventral horn (VH) and hindlimb muscle co-cultures. After locating functionally innervated muscle fibers, BoNT A or TeNT is added to the muscle compartment and these same fibers are assayed hourly for evoked muscle twitch. BoNT A eliminates evoked muscle twitch sooner than TeNT. Thus, whereas TeNT is more effective at spinal cord synapses, BoNT A is more potent at the neuromuscular junction, indicating differences in the efficiency of binding, internalization, and/or action of these toxins. Interestingly, BoNTs E and F have little effect on neurotransmitter release in spinal cord cell cultures, although they block evoked muscle twitch.

Dr. Lura Williamson has collaborated with Drs. Naveen Arora and Stephen Leppla (National Institute of Dental Research) and Dr. Jane Halpern (Food and Drug Administration) to characterize a chimeric protein consisting of anthrax lethal factor and TeNT light chain (LF-Tet) (Arora et al., 1994) or BoNT A light chain (LF-Bot). Spinal cord cultures are assayed to compare the effects of LF-Tet and LF-Bot with those of TeNT and BoNT A. Both TeNT and BoNT A holotoxins elicit differential effects on neurotransmission; i.e., inhibitory neurotransmitter release is blocked before excitatory transmitter release. In contrast, both LF-Tet and LF-Bot block inhibitory and excitatory neurotransmitter release at the same rate. No differential effects in neurotransmission are seen when the toxin light chains are internalized through a common alternate pathway suggesting that the differential effects on neurotransmitter release of TeNT and BoNT A are due to the differential distribution of receptors rather than internalization or substrate proteolysis.

Significance to Biomedical Research and the Program of the Institute:

The neurotoxicity induced by BoNT C might offer some advantages in therapeutic applications. Patients currently treated with BoNT A or F for muscle spasm disorders experience the recurrence of symptoms after a time interval depending on toxin serotype and the particular disorder. The limited effectiveness of toxin treatment may be due to the mounting of an antibody response, to the generation of axonal 'sprouts' and formation of new synapses, or to a return of function in blocked synapses following toxin degradation and substrate turnover. The action of BoNT C1 on two substrates of the synaptic vesicle docking/fusion complex might prolong time of recovery. More importantly, the inhibition of neurite outgrowth and synapse formation, and the severe neurotoxicity induced by BoNT C1 might prevent sprouting at the neuromuscular junction and ultimately lead to degeneration of the spastic neurons. Although further study is required in animal systems, BoNT C1 effects in neuronal cultures are strong indicators of its potential for clinical use.

Proposed Course:

Vesicle membrane recycling - We intend to demonstrate that vesicles recycled in the presence of BoNT A can be labeled with antibodies against synaptic vesicle proteins. We would like to repeat the BoNT A experiments using BoNT E, which also cleaves SNAP-25. However, since BoNT E is not effective in blocking neurotransmitter release in the spinal cord cultures, we will perform those experiments using nerve-muscle synapses in the multicompartment cultures. We will also obtain a chimeric toxin (BoNT A binding and transmembrane domains linked to the catalytic domain of BoNT E), which could be tested in spinal cord cultures.

BoNT C cytotoxicity - We will continue to test the hypothesis that BoNT C exerts its lethal effect via voltage-gated calcium channels by demonstrating a protective effect of calcium channel blockers. We plan to use HRP and colcemid to examine the origin of the vesicular elements found in swollen synaptic terminals in BoNT C-exposed cultures.

Rank order of toxin potency at the nerve muscle synapse - We will pursue comparative studies of the effects of the clostridial neurotoxins at the nerve-muscle synapse, and characterize the time course of action of selected toxins.

Toxin targeting - We will use multicompartment cultures to compare the intracellular movement of BoNT A with that of TeNT at the nerve-muscle synapse and in the axon of the motor neuron. Initially, we will track the toxins using fluorescence microscopy.

Gangliosides as toxin receptors - We will establish by thin layer chromatography that gangliosides are not present in cultures grown in fumonisin. We will use fumonisin-treated cultures to examine the role of gangliosides as functional receptors for BoNTs A, B, C, D, and G, in comparison with TeNT, to determine if gangliosides are uniformly necessary for toxin internalization.

Synaptic blockade and substrate proteolysis - We will correlate the kinetics of substrate proteolysis by toxin with inhibition of transmitter release in order to determine what proportion of substrate must be cleaved before neurotransmission is blocked.

Protocol:

94-012	Cell culture studies of neuronal development

Publications:

Halpern JL, Neale EA. Neurospecific binding, internalization, and retrograde axonal transport. In: Montecucco C, ed. Clostridial Neurotoxins. The Molecular Pathogenesis of Tetanus and Botulism. Berlin: Springer, 1995;221-41.

Neale EA, Bowers LM, Williamson LC. Vesicle membrane traffic at the synaptic terminal. In: Foster KA, ed. Biomedical Aspects of Clostridial Neurotoxins, in press.

Williamson LC, Halpern JL. Clostridial neurotoxin action in spinal cord neurons after uptake by an alternate pathway. In: Foster KA, ed. Biomedical Aspects of Clostridial Neurotoxins, in press.

Williamson LC, Halpern J, Montecucco C, Brown JE, Neale EA. Clostridial neurotoxins and substrate proteolysis in intact neurons: Botulinum neurotoxin C acts on synaptosomal protein of 25 kDa. J Biol Chem 1996;271: 7694-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
ZO1 HD 00708-12 LDN

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Morphologic Studies of Neuronal and Non-neuronal cells in CNS Cell Cultures

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.A. Neale	Head	LDN, NICHD
Others:	A.G. Parfitt	Adjunct Scientist	LDN, NICHD
	L.C. Williamson	Senior Staff Fellow	LDN, NICHD
	Q. Li	NRC Associate	LDN, NICHD

COOPERATING UNITS *(if any)*

Dept. of Physiology and Biophysics, School of Medicine, Case Western Reserve University (Maureen McEnery).

LAB/BRANCH
Laboratory of Developmental Neurobiology

SECTION
Section on Cell Biology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.35	0.35	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Multicompartment nerve-muscle co-cultures are used to study activity effects on synapse development and stabilization. Functional nerve-muscle synapses are identified by co-localization of activity-dependent uptake of FM1-43 with sites of alpha-bungarotoxin binding. Expression and distribution of Na^+K^+-ATPase isoforms and N-type calcium channel subunits appear to be regulated developmentally in spinal cord cell cultures.

Project Description:

Objectives:

To identify classes of cells, in cultures of nervous tissue, based on cell type specific proteins, neurotransmitters, neuropeptides, or receptors, in order to understand their interrelationships during development and to examine the morphologic development of neurons and phenomena related to neurite outgrowth and formation of early contacts with target cells.

Methods Employed:

Primary neuronal cell cultures, extracellular electrical stimulation, intracellular injection; morphologic techniques including light, video, fluorescence, and electron microscopy; computer-assisted image processing and analysis; immunohistochemistry.

Major Findings:

After extensive work to establish conditions that promote myoblast proliferation, Dr. Parfitt is able to provide three-compartment cultures in which myotubes are innervated by motor neuron fibers originating on opposite sides of the central myotube compartment. Innervation is demonstrated by stimulating electrically the nerve fibers that pass under the glass barrier between the compartments and observing muscle twitches that can be blocked with alpha-bungarotoxin. Exo- and endocytotically active portions of neurites intermingled with the muscle fibers are visualized using stimulus-mediated uptake and release of FM1-43 which should illuminate areas of synaptic activity. When cholinergic receptor patches on the myotubes are visualized with rhodamine-labeled alpha-bungarotoxin, however, it is clear that the majority of FM1-43 labeling in the neurites does not co-localize with these areas. This probably reflects the preponderance of non-cholinergic cells present in the tissue dissected from the ventral horn portion of the mouse spinal cord. FM1-43 labeling also extends over a considerable length of many neurites in the myotube compartment, in marked contrast to the punctate staining seen in the compartments which contain neurons only.

Dr. Lura Williamson is collaborating with Dr. Maureen McEnery (Case Western Reserve University) to examine the distribution and level of expression of the Na^+K^+-ATPase isoforms (alpha1, alpha2, and alpha3) by immunohistochemistry in developing and fully established spinal cord cell cultures. We find no staining of the alpha1 isoform in either developing or mature spinal cord cultures. Alpha2 immunoreactivity is specific to glial cells in all cultures examined. In contrast, alpha3 is specific to neurons and immunostaining is observed during synaptogenesis and through maturation of spinal cord neurons. Work from other laboratories has demonstrated the presence of all three isoforms in mature rodent brain. The spinal cord cultures mirror the isoform distribution *in vivo* and provide a reliable system for developmental studies. Additionally, we are performing a similar analysis for the alpha1B and beta subunits of voltage-dependent N-type calcium channels, for which there are no published data. Up to one week after plating, we find immunoreactivity for both alpha1B and beta subunits in neuronal cell bodies, but for only the beta subunits in neurites. In fully established neurons, both alpha 1B and beta subunits are localized in cell bodies and neurites, although immunoreactivity for the beta subunits is more intense.

Dr. Qun Li joined the laboratory on 1 August 1996 and has begun to learn techniques for the preparation of primary dissociated neuronal cell cultures from fetal mouse spinal cord for immunohistochemistry and intracellular dye injection, and for analysis of neuronal structure.

Significance to Biomedical Research and the Program of the Institute:

Ongoing electrical activity is critical for neuronal survival and for the formation and maintenance of appropriate neuronal circuits during early development. Activity effects are likely mediated by alterations in synaptic function. These studies, which allow the control of activity levels and the identification of synaptic structures, should increase our understanding of the relationship between neuronal structure and function, and provide insight into developmental disorders which result in loss of nervous function.

Proposed Course:

Electrical activity and neuron development - We plan to demonstrate that neuronal activity during development serves to optimize neuronal morphology for efficient input-output function. Experiments will examine the development of this morphology and quantify the effects of a total block in electrical activity, induced by either tetrodotoxin or tetanus toxin, on the early development of axons, dendrites, and synapses, identified immunohistochemically. If morphologic abnormalities can be demonstrated, we will examine the potential for reversal of effect. We will examine the fine structure of synaptic contacts that develop in the absence of activity. For comparison, we also will monitor preparations treated chemically (slightly elevated concentrations of KCl or low concentrations of tetanus toxin) to induce an increase in electrical activity. We will apply the same quantitative methodologies to the analysis of synaptic terminals in cultures incubated with protease inhibitors. These experiments relate to the phenomenon of synapse elimination and are described in detail under Dr. Nelson's Project # Z01 HD 00064-20.

Electrical activity and synapse elimination - We will continue with efforts to monitor morphologic changes that accompany synapse elimination from multiply innervated muscle fibers. Since the objective is to monitor changes in living cells, we must be able to identify relevant structures without fixing the cultures. We will attempt to identify synaptic terminals immunohistochemically using antibodies against components of the synaptic cleft, such as s-laminin or nerve-derived agrin. We will use fluorescent dyes to monitor calcium and/or voltage changes in the innervated myotubes following electrical stimulation of the neurites. (We hope, in this way, to establish the focus of initiation of the postsynaptic depolarization.) The third approach will be to identify, retrospectively, cholinergic nerve terminals using antibodies against choline acetyltransferase. Data from each of these techniques will be correlated with images from stimulus-mediated uptake of FM1-43 and alpha-bungarotoxin staining. Finally, we are attempting to increase the percentage of cholinergic fibers using spinal cord neurons derived from E-10 mouse embryos.

Development of N-type voltage dependent calcium channels - We will use double-label immunohistochemistry to examine alpha and beta subunit co-localization with synaptic terminal markers.

Protocol:

94-012	Cell culture studies of neuronal development

Publications:

Erickson J, Weihe E, Shäefer MK-M, Neale EA, Williamson L, Bonner TI, Tao-Cheng SJ-H, Eiden LE. The VACh/ChAT "cholinergic gene locus": new aspects of genetic and vesicular regulation of cholinergic function. In: Cuello CA, ed. Progr Brain Res, Amsterdam: Elsevier, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00710-08 LDN

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Characterization of Glutamate Receptor Expression in Brain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Buonanno	Head	LDN,NICHD
Others:	M. Sasner	IRTA Fellow	LDN,NICHD
	J. Cheng	Biologist	LDN,NICHD
	M. Ozaki	Visiting Fellow	LDN,NICHD

COOPERATING UNITS *(if any)*

Dr. J. Kusiak, NIH; Dr. D. Baird, Univ. Penn.; Dr. R. Feddersen, Univ. Minnesota

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Unit on Molecular Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.8	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Activity-dependent neural plasticity during development and in the adult is largely mediated by N-methyl-D-aspartate receptor (NR) activation. Glutamate is the natural ligand for this excitatory amino acid receptor that is expressed specifically in the CNS. The functional properties of NRs have been found to change in the cerebellum and visual cortex during the development; the changes in the visual system are activity-dependent. Using in situ hybridization, we have found that the down-regulation of NR2B and increase of NR2C mRNA levels in the internal granule layer of the cerebellum closely follow the spatial-temporal pattern of synapse formation, suggesting that these NR genes are regulated by innervation. Using cerebellar slice cultures, we have found that neurotrophic factors specifically up-regulate NR2C mRNA levels with little effects on NR2B expression. On the other hand, preliminary evidence suggests that the down-regulation of NR2B in granule cells during synaptogenesis may result from activity. Thus, different NR subunits may not only function to distinctly modulate synaptic connections in response to activity, but their expression patterns may also be differentially responsive to epigenetic factors. In order to understand the complex mechanisms that direct regional-specific transcription of NR genes during neurogenesis and modulate their levels in response to synaptic activity, we are investigating the mechanisms that control expression of the NR2 subunits at the transcriptional level. Utilizing transgenic mice, we have identified different upstream regions of the NR2B gene that are required for its neural-specificity and its developmental down-regulation in the cerebellum. We have recently succeeded transfecting cultures of primary cerebellar granule cells with NR2B reporter constructs, which has enabled us to delineate sequences that regulate NR2B transcription. Deletional and mutational analyses are in progress to identify the cis-acting sequences that mediate neural-specific transcription of the NR2B gene in cultured cells and in the brain of transgenic mice. In collaboration with Dr. Kusiak, we are using transgenic mice to analyze NR 1 DNA regulatory sequences that confer neural specificity; the DNA constructs were previously shown to function in transfected PC12 cells. In preliminary experiments we found that the upstream 3.0 kb of the NR1 gene directs expression of the beta-gal transgene to most CNS neurons. The NR cis-acting elements identified in these studies will be used to isolate novel transcription factors that confer neural specificity and mediate the effects of electrical activity.

Project Description

The changes in synapse efficacy during development and in the adult are dependent on NMDA receptor (NR) activity, and are accompanied by differences in the electrophysiological and pharmacological properties of the receptor. The molecular basis for the differences in receptor properties are mostly unknown, with exception of the cerbellum, where differential expression of NR subunits coincide with the electrophysiological properties of the receptor. During development, migrating granule cells that express the NR 2B gene turn expression off in the internal granule cell (IGL) layer and begin to express the NR 2C subunit. This switch in subunit expression is followed by changes in the single channel properties of the receptor. In contrast to the cerebellum, expression of the 2B gene peaks at P14 in the forebrain during the period of maximal synaptogenesis and continues to be expressed thereafter. Since the receptor composition can influence the amount of calcium entering the cell and the downstream signaling that leads to neural plasticity, we are interested in understanding the molecular mechanisms that regulate the expression of the 2B and 2C subunits during cerebellar development. In particular, we are interested in the effects of innervation (and activity) on the down-regulation of NR 2B in cerebellum and the up-regulation of the 2C subunit.

Objectives:

1) To characterize the DNA regulatory sequences that confer neural and developmental-specific transcription to the NR 2B gene; specially those regulating the activity dependent down-regulation of the gene.

2) To identify the cellular mechanisms that regulate the NR 2B to NR 2C exchange in developing cerebellum.

Methods:

Cloning and characterization of NMDA receptor genes : Cloning of the mouse NR 2B gene were described in last year's report. The NR 2C gene was isolated from the same lambda DASH-1 library. The genomic inserts were mapped on Southern blots using specific oligonucleotides. DNA sequence of subcloned fragments was obtained by the Sanger dideoxy-termination technique using oligonucleotides as primers.

Generation of transgenic mice and analyses : Transgenic mice were generated by injection of (C57BL/6 X SJL)F1 or FVB/N pronuclei with linearized DNA containing mouse 2B and 2C gene upstream sequences linked either to the CAT or β-gal reporter genes. The construct 0.8-CAT is a 806bp region extending from the HindIII to the SacII site which contains the first exon and upstream sequence. 0.8int-CAT harbors a fragment between the same HindIII site and the end of the second exon. The region -552 to +255 (0.8-CAT) and the region -552 to +1631 (0.8int-CAT) were cloned into the HindIII-XbaI sites upstream of the CAT gene in the pCAT-Basic vector (Promega). Three CAT-expressing lines (#6184, 6188, and 6190) for the 0.8-CAT construct and two CAT-expressing lines (#6944, 6948) for the 0.8int-CAT construct were characterized. To analyze the NR1 regulatory sites, mice were injected with a β-gal expression vector driven by the upstream 3.0 kb region. F1 animals were tested for incorporation of the transgene by slot blotting DNA prepared from tail clips. Slot blots were also used to determine transgene copy number. The tissue-specificity conferred by the regulatory sequences was determined by assaying for CAT activity in extracts made from different tissues or staining for β-gal activity using X-gal as the chromophore. A time course was performed to show that these conditions are in the linear range of the assay. Quantitation was performed on a Molecular Dynamics phosphorimager system.

Major Findings

Although there has been considerable progress in understanding the molecular mechanisms regulating the tissue and developmental expression of genes in non-neuronal cells, this advance has not been evident in the nervous system. The bewildering complexity of neural and glial cell types in the nervous system, the intricate connections between cells and the added disadvantage that genetic material is difficult to introduce into the post-mitotic neurons (see below), greatly accounts for the lack of systems suitable for studying genetic regulation in neurons. The cloning of cDNAs coding for NR subunits represented the first step in understanding the molecular composition of native receptors. Recent studies strongly support the notion that hetero-oligomerization, between the widely expressed NR1 subunits and members of the NR 2A-D family, is necessary to form receptors with appropriate electrophysiological properties. The composition of native receptors largely depends on the regional and temporal expression of the NR2 subunits. The kinetic properties of NRs in cerebellar granule cells change during development; migrating granule cells (prior to innervation) express receptors that have higher conductance, longer mean open channel times and burst lengths than the post-migratory granule cells residing in the IGL of P22 rats (which probably have received afferent inputs). A switch to receptors with lower conductance and/or mean open channel times during development would presumably lower Ca^{2+} influx, in this fashion regulating signal transduction and neural plasticity. Granule cells present in the EGL and those that have migrated into the IGL express NR 1 and NR 2B, whereas by P14, most granule cells have begun to express the NR 2C transcripts and to down-regulate NR 2B expression. The single channel properties of the pre-migratory and maturing granule cells closely resemble those of in vitro synthesize dimeric receptors NR1/NR 2B and NR1/NR 2C, suggesting that the regional/temporal regulation of subunit gene transcription ultimately controls receptor function. The change in NR subunit expression is reminiscent of the γ to ε switch that occurs at the neuromuscular junction during muscle innervation and maturation. In this case, neural activity down-regulates γ subunit gene transcription and neurotrophic factors up-regulate the expression of the ε subunit. As discussed below, we have preliminary evidence that suggest that similar cellular mechanisms are utilized to control the expression of NR subunits during granule cell maturation. We and others have evidence that the NR 2B gene, like the acetylcholine receptor γ subunit, is down-regulated by synaptic activity, and that NR 2C expression is up-regulated by neurotrophic factors. Considering that the various combinations of NR1 with the different NR2 subunits impart to the receptor different channel properties, it is evident that comprehending how NR2 subunit expression is regionally and developmentally regulated would contribute to our understanding of the molecular mechanisms underlying neural plasticity.

Mechanisms affecting the NR 2B to NR 2C exchange in cerebellum. Using *in situ* hybridization, Dr. Buonanno previously found that the expression of NR 2B and 2C transcripts differs markedly in the external (EGL) and internal granule cell layers (IGL) during cerebellar development. Pre-migratory granule cells in the EGLexpress NR 2B, by P14 levels become undectable in the IGL. In contrast, during postnatal days 8 and 21, NR 2C transcripts accumulated in a posterior/anterior and medial/lateral gradient in granule cells located exclusively at the IGL. These expression patterns suggested to us that transcription of the NR 2C gene is up-regulated during synaptogenesis whereas NR 2B is repressed. Similar to the acetylcholine receptor γ to ε subunit switch that occurs during skeletal muscle development, our findings show that the initial NR2B expression in granule neurons is cell-autonomous, whereas innervation may provide the signals that activate NR 2C expression and repress NR2B. Preliminary experiments to address these issues have been performed in dissociated primary granule cell cultures, cerebellar slice cultures and transgenic mice.

Dr. Sasner has analyzed the effects of NMDA receptor antagonist and neurotrophic factors on the relative levels of NR 2B and 2C transcripts in dissociated granule cell cultures using quantitative PCR. In these conditions, the NMDA receptor antagonist APV was found to up-regulate the levels NR 2B transcripts and neurotrophic factors did not affect levels of the NR 2C subunit. In contrast, in cerebellar slice cultures we found that neurotrophic factors have the capacity to increase NR 2C levels; the group of Dr. Audinat has reported that NR 2B levels increase in slices when activity is blocked. Taken together, these experiments suggest that activity is sufficient to down-regulate NR 2B expression, but that neurotrophic factors are not sufficient to increase NR 2C expression and require cell-cell interactions that are provided in the slice preparation. Are the anterograde signals provided by mossy fiber inputs or the retrograde signals from Purkinje cells important for the regulation of NR subunits? Utilizing transgenic mice we have analyzed the latter question. In collaboration with Dr. Rod Fedderson, who has generated a line of transgenic mice that produce Purkinje cells which fail to generate dendritic arbors, we have analyzed the regulation of the NR 2B and NR 2C subunits during development. Using both Northern blot and in situ hybridization we have failed to find temporal differences in the regulation of either the NR 2B or 2C subunits, suggesting that retrograde signals are not sufficient to regulate the 2B/2C subunit exchange. Experiments are in progress to analyze the role of mossy fiber inputs in the regulation of these NR subunits.

Transcriptional regulation of the NR2B gene. During the past 2 years, Dr. Sasner has cloned, sequenced and analyzed the regulation of the NR 2B gene. In these studies he identified a 0.8 kb region (from -552 to +225) that is sufficient to confer tissue-specificity in transgenic mice, and a distinct region containing the first intron and second non-coding exon (from -552 to + 1631) that is responsible for the developmental down-regulation of the NR 2B gene in the cerebellum. Sequence for regulatory elements that have previously been shown to bind brain-specific transcription factors were found within these NR 2B regions (published in the Journal of Biological Chemistry). These results have enabled us to begin studying the cis- and trans-acting elements that control both the tissue-specificity of the gene and its developmental down-regulation in the cerebellum. A series of transgenic mouse lines were generated with NR 2B constructs harboring different deletions in the upstream 0.8kb region and the first intron. Mice harboring a CAT construct missing the region between -552 and -255 continue to express the transgene specifically in the brain, indicating that elements conferring neural specificity reside between -255 and +225 bp. Interestingly, lines harboring a series of deletions that progressively remove most of the first intron continue to properly down-regulate the CAT reporter gene in developing cerebellum, indicating that elements residing in the second non-coding exon are important for NR 2B repression in the adult cerebellum.

A significant technical break-through was made this year with the modification of a transfection protocol that enabled us to transfect cerebellar granule neurons. Utilizing this technique, Dr. Sasner has transfected granule cells with luciferase (luc) reporter constructs driven by NR 2B regulatory sequences. Luciferase activity in granule cells transfected with the construct containing the 0.8kb NR 2B upstream regulatory region is approximately 25-fold higher than in cells transfected with a promoter-less vector or 5-fold higher than cells harboring a construct driven by the SV40 promoter + enhancer. Transfection in non-neural cells confirm that the upstream NR 2B sequence also confers neural specifity in vitro. Dr. Sasner has also used the transfected granule cells to demonstrate that the NR 2B upstream sequence confers responses to activity. Experiments are in progress, using the transfection assays in cultured cells and transgenic mice, to identify the regulatory sequences conferring neural specificity and the down-regulation of the NR 2B gene during cerebellar development.

Characterization of the NMDA receptor NR 2C gene. We are interested in developing molecular markers to analyze how the NR 2B gene is repressed by granule cell innervation, and replaced by expression of the NR 2C. To this end, we have generated antisera against the NR 2C subunit and have isolated the gene. Experiments to fully characterize the specificity and utility of the antisera for immunohistochemistry are in progress. In collaboration with Dr. D. Baird, we are co-culturing pontine neurons with their post-synaptic cerebellar granule cell targets to determine if innervation up-regulates NR 2C expression. We are also generating transgenic lines harboring β-gal constructs driven by 5'-flanking NR 2C sequences to determine how presynaptic inputs regulate this gene.

Significance to Biomedical Research and the Program of the Institute:

Glutamate receptors are known to mediate excitatory postsynaptic transmission in the brain, and are the most abundant excitatory receptors in the vertebrate CNS. Glutamate and its analogs are potent neurotoxins. In addition, experimental animal studies have suggested that GluRs maybe implicated in some degenerative phenomena in the CNS, such as Huntington's disease. In fact, intrastriatal injections of kainate produce a pattern of neuronal degeneration and biochemical changes similar to those observed in postmortem tissue from Huntington's disease patients. An important step in understanding the basis of neurotoxicity, and the expression of GluRs during development, is to identify the genes coding for these receptors. The analysis of how GluRs are expressed and regulated during cerebellar development at a genetic level will set some of the foundations for the understanding of the developmental biology of glutamatergic synapses; sites for the action of several psychoactive drugs.

On the other hand, the delineation of regulatory sequences that temporally and regionally direct gene expression in the brain is of extreme importance for their future use in gene therapy. Presently, there is a notable absence of characterized promoters directing neural expression in specific regions of the brain. Current experiments on the use of gene therapy in the CNS generally utilize expression constructs that are driven by viral promoters; these promoters have the disadvantage that they lack specificity. The present approaches available to introduce the expression constructs into the CNS rely on the stereotaxic delivery of recombinant viral vectors (i.e. herpes simplex virus or adenovirus) or genetically modified neuronal cells harboring the expression constructs; the former strategy has the advantage of avoiding cellular immunological responses. However, the level of specificity provided stereotaxic injections of either viruses or cells is minimal. The use of developmental- and regional-specific neural promoters in the expression constructs would greatly improve the specificity of these approaches. An obvious requirement in the use of neural-specific promoters to target expression is that the viral vectors have a size limitation. For this reason, it will be imperative to delineate the shortest DNA regulatory sequences providing specificity and quantitative expression. Our work on the characterization of NR regulatory sequences provide an initial step toward this goal.

Proposed Course:

Dr. Sasner will continue to analyze the regulatory sequences that impart neural- and developmental-specific expression of the NR 2B gene in cultured cells and transgenic mice. We are presently generating transgenic lines that harbor constructs containing deletions in the NR2B gene second non-coding exon in order to locate the cis-elements conferring down-regulation of the gene during development. In collaboration with Dr. P. Nelson's group, we plan to use cultures derived from the transgenic mice and transfected granule neurons to study how NR transcription is repressed by electrical activity. We will continue work with the transfected granule cells and transgenic mice to identify the cis-acting element(s) conferring neural-specificity to the genes encoding the NR 1 (in collaboration with Dr. Kusiak) and NR 2B subunits.

Drs. Buonanno and Sasner will continue to use the slice preparation to determine the mechanism of neurotrohic factors on NR 2C expression, and in collaboration with Dr. Baird we plan to determine the cell types required for this gene's activation.

Protocols:

Animal:

93-029	Buonanno	Targeted DNA delivery into tissues
93-030	Buonanno	Production of transgenic mice

Publications

Sasner M, Buonanno A. Distinct NMDA 2B subunit gene sequences confer neural and developmental specific expression. J Biol Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00711-07 LDN

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transcriptional Regulation of Skeletal Muscle-Specific Genes by Electrical Activity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Buonanno	Head	LDN, NICHD
Others:	S. Calvo	Adjunct Scientist	LDN, NICHD
	M.Nakayama	IRTA	LDN, NICHD
	J. Stauffer	IRTA	LDN, NICHD
	J. Cheng	Biologist	LDN, NICHD

COOPERATING UNITS *(if any)*

Dr. E. Wawrousek (NEI, NIH); Dr. J. Weis (Univ. of Aachem, Germany)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Unit on Molecular Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20982

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.8	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our long-term interest is to understand at a molecular level how motor circuits are established and maintained. We would like to understand how motoneurons are initially matched with their corresponding types of myofibers (targets) during embryonic development, and later, how the contractile properties of the muscle continue to be regulated by motoneuron depolarization. Nerve-elicited activity initiates signal transduction cascades that culminate in the nucleus, where distinct frequencies of depolarization differentially regulate the transcription of genes encoding fast- or slow-twitch contractile proteins and leads to the down-regulation of receptors transcribed at extra-junctional nuclei. We have used the regulation of troponin I slow (TnIs) and fast (TnIf) isoforms as models to study activity-dependent transcriptional regulation; both of these genes are differentially up-regulated by specific depolarization frequencies (100 vs. 10 Hz). Because activity-dependent fiber specification is not observed in cultured muscles, we have used transgenic mice to identify the regulatory sequences that direct TnI transcription to either slow or fast-twitch myofibers. Using this approach, we identified a 128bp rat TnIs slow upstream regulatory element (SURE) and a 144bp quail TnIf fast intronic regulatory element (FIRE) that direct transcription of the chloramphenicol acetyltransferase (CAT) transgene specifically to either slow or fast muscles, respectively. Sequence alignment of the rat and human TnIs SURE with the quail TnIf FIRE identified common DNA motifs, namely two A/T-rich sequences (A/T1 and A/T2) with homology to homeotic protein and MEF2 binding sites, a CACC box, an E box, and a novel motif (GCAGGCA) that we denoted the CAGG box. Point mutations in either the A/T2 site, E box or CAGG box practically abolish the SURE function; mutations in the A/T1 and CACC sites have a lesser effect. Using competitive electrophoretic mobility shift assays with nuclear extracts derived from Sol8 myotubes, we demonstrate specific binding to these motifs and show that the A/T1 and A/T2 bind different factors. Our results demonstrate that the linear arrangement of DNA sequence motifs is conserved in the regulatory elements of the TnI slow and fast genes, and suggest the interaction of multiple protein-DNA complexes are necessary for enhancer function. In the past years, we have investigated the possible role of the myogenic transcription factors MyoD, myogenin and MRF-4 in the regulation of acetylcholine receptors and contractile proteins. We have found that these factors are not expressed selectively at junctions nor specific fiber-types, and that denervation dramatically increases their expression levels (10-100 fold). An upstream region in the myogenin gene of approximately 400 bp has been mapped that is required for its denervation-dependent transcriptional activation. Sites binding immediate early genes present in this region are being investigated.

Project Description:

Objectives:

1) To identify the transcription regulatory sequences in the slow and fast troponin I (TnI) genes that confine gene expression to slow- or fast-twitch muscles of transgenic mice.

2) To characterize and isolate the transcription factors that bind the motifs in the SURE and the FIRE, and which ultimately regulate the expression of contractile genes in response to specific frequencies of electrical activity.

3) To delineate sequences in the myogenin promoter that confer innervation-dependent regulation in skeletal muscle.

4) To analyze the regulation of the myogenic factor MRF-4 in denervated muscle.

Methods:

Characterization of TnI regulatory sites in transgenic mice: The cloning and initial characterization of the rat TnIs gene was described in our previous Annual Report. Nested deletions in sequences residing in the upstream region were generated from both directions using ExoIII and S1 nuclease. DNA sequencing was performed using standard di-deoxynucleotide termination reactions. GeneWorks software (IntelliGenetics, Inc.) was used for the sequence and alignment analysis.

Transgenic mice were generated essentially as described by Hogan et al. [Hogan, Beddington, Constantini and Lacy (1994) Manipulating the Mouse Embryo, Cold Spring Harbor Press]. Transgenic mice were generated in (C57Bl/6 X SJL) F1 or FVB/N embryos. A series of TnI slow constructs containing different amounts of the upstream sequences were used to generate transgenic lines. Putative founders and their generations were screened by slot blot analysis of tail DNA using a CAT probe. Adult transgenic mice were used to analyze tissue- and muscle-type-specific expression of CAT activity. Tissue-specificity was determined by measuring levels of CAT activity in extracts made from tissues that either express or do not express the endogenous TnI slow gene. In order to further determine if fiber-type-specific expression was conferred by the constructs, muscle groups known to contain different proportions of fast and slow myofibers were analyzed for CAT activity.

Characterization of myogenin gene transcription regulatory elements in muscle: The generation of transgenic mice harboring chloramphenicol acetyltransferase (CAT) expression vectors driven by the mouse myogenin gene upstream regulatory sequences was described on the previous Annual Report. We analyzed mice harboring 3.7, 1.5 and 1.0 kb of upstream sequence. The tissue, developmental and innervation specificity conferred by the regulatory sequences was determined by assaying for either CAT transcripts or enzyme activity. We analyzed for innervation responsive elements in the construct by comparing CAT mRNA levels in denervated and innervated hind limb muscles; these were denervated by cutting the sciatic nerve at the upper thigh. The relative CAT mRNA levels were quantitated by RNase protection and Northern blots using the Molecular Dynamics phosphorimager.

Major findings

Introduction:

Skeletal muscles develop from post-migratory committed cells that differentiate, and by two successive waves of fusion generate the primary and secondary population of myotubes which become multiply innervated by alpha motoneurons. As development proceeds, the fibers mature and diversify into the distinct slow- and fast-twitch extrafusal fibers present in the mature muscle, and poly-innervation is eliminated resulting in myofibers receiving single inputs from alpha motoneurons. During this process of myofibril maturation, the distribution of receptors and the types of contractile protein isoforms expressed in embryonic myotubes change with motoneuron innervation: nicotinic acetylcholine receptors (nAChRs) expression is repressed extra-junctionally, ε-containing nAChRs accumulate specifically at the neuromuscular junction, and expression of adult isoforms of contractile proteins is restricted to slow and fast-fibers. Although the adult muscle is composed of fully differentiated myofibers, its properties are remarkably plastic. Muscle retains the capacity to modify its contractile and metabolic properties in response to changing demands on force generation and energy utilization, humoral factors, and especially to different patterns of neural derived electrical activity. Denervation of skeletal muscle causes muscle to re-adopt its embryonic properties; for example, extrajunctional "embryonic-type" nAChRs increase by 100 to 1000-fold and the embryonic/fetal isoforms of myosin heavy chain (MHC) are reexpressed. Furthermore, the contractile properties of adult muscles can be modified by innervation patterns. Fast-twitch muscles adopt slow-twitch properties when they are re-innervated ectopically by a nerve that normally innervates a slow muscle, and vice-versa. Depolarization, and not myotrophic factors released by the nerve, is thought to be instructive to muscle since electrical stimulation of denervated muscles with patterns that mimic natural motoneuron activity are sufficient to modify its contractile properties.

One approach to understanding the molecular mechanisms controlling muscle plasticity and diversification is to identify the cis- and trans-acting regulatory factors that mediate the activity response. The long-term goals of our laboratory is to identify factors that transduce electrical signals from the motoneuron to changes in gene expression in the nucleus, and in this fashion regulate muscle plasticity. As model systems we have analyzed the effects of specific frequencies of electrical activity on the regulation of the MyoD-related myogenic transcription factors and the fiber-specific expression of the troponins I slow (TnIs) and fast (TnIf). Although there has been enormous progress in the identification of factors that regulate muscle determination and commitment, the most noted are members of the MyoD and MEF-2 families of transcription factors, little is known about the factors that regulate muscle plasticity and diversity. The fact that the mature properties of muscle do not develop in vitro and require to be analyzed in vivo, in great part accounts for the lack of understanding of which factors are important for regulating adult muscle properties. To this end, we have used transgenic mice to study gene regulation in embryonic, fetal, mature and denervated muscles. Previously we found that the MyoD family of myogenic transcription factors, which include MyoD and myogenin, participate in the re-establishment of an embryonic program after the denervation of adult muscle; the function of these factors in innervated muscle has not been demonstrated. During this year, we have extended our studies to the regulation of MRF-4. In contrast to MyoD and myogenin, which are dramatically down-regulated during myofibril innervation, MRF-4 mRNA levels increase during muscle maturation; this suggested its putative participation in regulating adult fiber-type properties. In collaboration with Dr. Joaquim Weis at the University of Aachem in Germany, we have analyzed MRF-4 expression during innervation, after denervation and its distribution in distinct fiber-types of rat adult muscles. From our previous studies on myogenin and MyoD, and these studies on

MRF-4, we conclude that this family of transcription factors is not sufficient to regulate the contractile properties of muscle in response to different patterns of electrical stimulation and distinct factors are necessary (see below). Whereas depolarization down-regulates the expression of MyoD-related factors independently of frequency, the up-regulation of TnI expression is selectively controlled by different patterns of electrical stimuli. These experiments suggested to us that there should be at least two innervation-dependent regulatory cascades that differentially modulate the mature properties of fiber-types. Our studies of the TnI regulatory elements in transgenic mice has yielded important information on this regard. As discussed below, we have identified TnI enhancers that have the capacity to restrict transcription to either slow- or fast-twitch fibers. Mutational analysis of these enhancers demonstrate that complex interactions of factors binding to conserved cis-acting elements are required for the fiber-type specific expression of TnI genes. Experiments are in progress to identify the trans-acting factors that confine TnI transcription to either slow or fast muscles in response to specific patterns of motoneuron activity.

MRF-4 protein is detectable in denervated but not in innervated muscle. In collaboration with Dr. Joaquim Weis, Drs. Soledad Calvo and A. Buonanno have analyzed the expression of MRF-4 protein in the innervated and denervated muscles of adult rats. A specific polyclonal antiserum was generated by immunization of rabbits with of a GST fusion protein containing a carboxy-terminal region of MRF-4 which is not conserved with the other myogenic MyoD-related factors. The antisera was shown to be specific by immunohistochemical analysis of cells transfected with different myogenic factor expression constructs. In contrast to numerous other antisera developed against MyoD-related proteins, it reacts in fresh-frozen sections of adult muscle as well as on Western blots. Utilizing a series of techniques, we found that MRF-4 is the first factor to accumulate in the nuclei of denervated muscles. However, utilizing a series of different detection techniques, we were unable to detect MRF-4 immunoreactivity in adult muscles. Immunoreactivity is confined to myofribril and satellite cell nuclei, and does not show preference for fiber-type. These results, in addition to our previous findings that MyoD and myogenin are not detected in innervated muscles, suggest that translation may be an important regulatory mechanism for controlling factor expression in innervated fibers. Denervation leads to increases in mRNA and protein, consistent with our hypothesis that MRF-4, like MyoD and myogenin, plays a role in the reactivation of selective genes normally expressed in embryonic muscle. The fact that the appearance of MRF-4 protein precedes by over 24 hours the increase in myogenin and MyoD expression in denervated muscle, could suggest that MRF-4 may participate in the regulation of the latter two factors (this manuscript is in review).

Common core elements are shared by the TnI enhancers conferring specific expression in either slow and fast muscles: We have used the regulation of the TnI genes as a model to elucidate the mechanisms that generate fiber diversification in response to epigenetic factors. Transcription of TnI genes is activated during the myoblast terminal differentiation. During muscle maturation, the expression of the two TnI isoforms is confined to either slow or fast muscles in response to the type of moto-innervation the myofibers receive. As we previously demonstrated, specific patterns of electrical activity that mimic the endogenous frequencies of fast (100 Hz) and slow (10 Hz) nerve regulate TnI gene expression selectively. Because fiber-specification is not observed in cultured mammalian muscle, Drs. Manabu Nakayama, Jim Stauffer, Soledad Calvo and Ms. Jun Cheng have used transgenic mice to identify regulatory sequences that direct the fiber-specific transcription of the slow and fast TnI genes. Multiple lines of transgenic mice were generated that harbored reporter constructs driven by systematic deletions of the upstream regions of the rat TnIs gene. Mice harboring chloramphenicol acetyltransferase (CAT) reporter constructs, driven by approximately 1900 and 950 bp of the rat TnI slow gene upstream sequence, are transcribed specifically in slow muscle, but not in fast muscles or other tissues such as heart, liver or brain. In contrast, mice harboring constructs containing the upstream TnI slow 250 or 500 bp failed to be expressed in embryonic or adult skeletal muscle. Fiber-type-specific transcription in slow muscle was restored when a 128 bp slow upstream regulatory enhancer (SURE), that is located between the 950 and 500 bp upstream region of the gene, was fused to the 500 bp fragment. In order to test if the transcription specificity is conferred by the 128 bp enhancer and not the 500 bp region, we generated transgenic mice with a construct containing the 500 bp promoter fused to an intronic regulatory region derived from the quail Tn I fast gene. This 144 bp sequence was previously shown to function as an enhancer in differentiated cultured myotubes. Mice harboring the chimeric TnI fast/TnI slow CAT construct specifically expressed the reporter gene in fast but not in slow muscles, indicating that these 2 enhancers selectively direct fiber-type-specific transcription when fused to a common TnI slow 500 bp promoter region. As shown in Figure 1, alignment of the rat and the human TnI SUREs and the quail TnI fast intronic regulatory enhancer (FIRE) showed that these enhancers share similar core elements, namely two A/T-rich sequences (A/T1 and A/T2) with homology to homeotic protein and MEF2 binding sites, a CACC box, an E box, and a novel motif (GCAGGCA) that we denoted the CAGG box (manuscript published in Molecular and Cellular Biology).

```
                            A/T1 box                CAGG box
RAT SURE     -859   TGACCGACTATAATAGCTACCGGATTAACATAGCAGGCAT--TGTCTTTCT
                    |||| |||||||||||| || |||||||||||||||||||  |||||||||
HUMAN SURE   -989   TGACAGACTATAATAGCCACAGGATTAACATAGCAGGCAT--TGTCTTTCT
                    |  | ||  | |       | |||  ||||   |||||||       || ||
QUAIL FIRE   +776   TCCCTGAGGAAACCTTATCCTGGA-TTAAATGT-GCAGGCACAACACATTGCT


                                CACC box                        A/T2 box
RAT SURE            CTGA----CTATAGGGTGGGTATTATGTGTTCATCAACAGTCCTAAAAATA
                    ||||    ||||||||||||||||||||||||||||||  |||||||||||
HUMAN SURE          CTGA----CTATAGGGTGGGTATTATGTGTTCATCAACCATCCTAAAAATA
                      ||    | |  |||||||      | |   |  |    | ||||||||
QUAIL FIRE          GGGAAGAGCAAGGGGGTGGG------GGGGGAA--AGTGCTTCTAAAAATG

                                           E box
RAT SURE            CCCAGG-AA-------------ACAGGTGTGGCCC-TG   -741
                    ||| || ||             |||||||  |||| ||
HUMAN SURE          CCC-GGTAA-------------ACAGGTGCAGCCCCTG   -870
                     |  || |               ||| ||   || | |
QUAIL FIRE          GCTGGGAGCTGCACAAGGAGCTGCAGCTGTCTCCTCAG   +645
```

Figure 1. Core elements are conserved between the rat and human TnI slow upstream regulatory region, and the quail TnI fast intronic regulatory region. Sequences from the sense strands of the rat and human TnI SURE and the non-sense strand of the quail TnI FIRE have been aligned; the location of these regions is numbered with respect to the transcription start site of each respective gene of origin. The common core elements between the sequences are in bold face characters. These include consensus binding sites labeled as the CACC box, A/T2 box, E box and the novel CAGG box found in the human and rat SURE, and in the quail FIRE. Additionally an A/T-rich motif conserved between the rat and human TnI SURE is in bold face and labeled as the A/T1 box.

We previously demonstrated that regulatory sequences also reside in the rat TnIs second intron (that cooperate with the upstream enhancer to increase transcription [Banerjee-Basu and Buonanno, 1993]. A 125 bp sequence was identified in the intron that shared part of the motifs also found in the SURE and FIRE, specifically a CACC motif and E box. Our resent studies demonstrate that a small deletion of part (56 bp) or the full (125 bp) conserved region, in the context of the full length 4.3 kb construct (from -479 to + 3800), reduces transcription by over 50% in transfected Sol8 myotubes. These results demonstrate that the TnI slow intronic regulatory element (SIRE) is necessary for the higher reporter levels observed in myocytes transfected with the full-length 4.3kb construct.

Functional analysis of elements regulating fiber-type-specific transcription: The functional role of the different conserved motifs in the rat TnI SURE were studied by mutational analysis in transfected myocytes and transgenic mice, and by DNA/proteins interactions using electrophoretic mobility shifts assays (EMSAs). Thus far we have analyzed in transgenic mice the TnI SURE function in mice harboring CAT reporter constructs with point mutations in either the CACC site, E box or CAGG box. Any of these mutations abolish SURE function. In transfected Sol8 myotubes mutations of either the A/T2 site, E box or CAGG box also eliminate the SURE function, whereas mutation of the A/T1 and CACC sites have a lesser effect. Using competitive EMSAs with nuclear extracts derived from Sol8 myotubes, Dr. Stauffer has demonstrated specific binding to these motifs. The A/T1 and A/T2 sites are shown to form different complexes. The A/T2 site, which bares extensive homology to a MEF2 site, forms complexes that are super shifted by MEF2A antisera and that are competed by a consensus MEF2 site present in the MCK enhancer. Our results demonstrate that the linear arrangement of DNA sequence motifs is conserved in the regulatory elements of the TnI slow and fast genes, and suggest the interaction of multiple protein-DNA complexes are necessary for enhancer function. Experiments are in progress to identify and isolate the factors conferring the fiber-type-specific expression of the TnI genes in response to activity (manuscripts published in Developmental Genetics).

Characterization of the myogenin gene regulatory sequences: Our previous work has strongly suggested a causal link of nAChR gene regulation by the MyoD-related factors, specially the innervation-dependent transcriptional regulation of receptor genes by myogenin at extra-junctional nuclei (see Previous Reports). To elucidate the molecular pathways linking the effects of muscle depolarization with the repression of gene transcription we have begun to characterize the regulatory elements in the myogenin gene, since its regulation by innervation is more proximal. In previous studies, where myoblast implantation was used to study gene regulation in vivo (see Annual Report '95), we found that mutation of sequences residing between -1.5 and -1.1 kb upstream of the myogenin transcription site caused a temporal shift in the activation of myogenin transcription after denervation. In order to map the sites that are required for the early activation of myogenin, transgenic mice containing 3.7, 1.5 and 1.1kb of myogenin upstream sequence driving the expression of the CAT reporter gene were generated and analyzed by Dr. Soledad Calvo and Ms. Jun Cheng. RNase protection assays for CAT message were performed, instead of CAT assays, in order to detect temporal changes in transcription rates more precisely. As first seen with myoblast implantation, we found that sequences between -1.5 and -1.1kb are necessary to recapitulate the temporal time-course of myogenin accumulation after denervation; constructs missing this sequences increase denervation-dependent gene transcription after 48 hours instead of 16 hours. Interestingly, this region contains DNA motifs that are similar to immediate early gene binding sites. Experiments are in progress to delineate the cis-acting elements necessary for this early response elements, and to determine if they are necessary for the activity-dependent repression of myogenin.

Significance to Biomedical Research and the Program of the Institute:

The long-term goal of my research is to understand at a molecular level how motor units are established and maintained. In other words, we would like to understand how motoneurons are initially matched with their corresponding types of myofibers (targets) during embryonic development, and later, how the contractile properties of the muscle continue to be regulated by motoneuron activity. This area of work is not only important for our general understanding of how motor circuits are generated during development, but also provides information of general relevance for clinical studies on patients that have suffered spinal chord lesions or neuropathies where neural-muscular connections have been compromised. The work carried out in our Unit is of general interest to the program of NICHD, since it seeks to understand the cellular and molecular mechanisms underlying muscle development. The identification of myotrophic factors would be of obvious interest to the clinical program, as well as information concerning satellite cells during regeneration. There has been an increasing interest that myoblast implantation may serve as an exciting form of gene therapy for immunization, hormone delivery and the treatment of patients with muscular dystrophy, where repopulation of muscle with myoblasts carrying a healthy dystrophin allele is sufficient to restore muscle function. However, up to date there has been limited success in the initial clinical trials performed since only a small fraction of the myoblasts implanted survive for long periods. It has been proposed by several investigators that success in this area may be difficult without a deeper understanding of the diversity that may exist among satellite cells (experiments performed in cats over a decade ago suggest this possibility) and of the neural-derived myotrophic factors that are required for the maintenance of muscle.The identification of regulatory elements that determine muscle specification during development, is specially important for its use in targeted gene therapy. Our myoblast implantation experiments demonstrate that these regulatory regions function in adult muscle, and show that gene products are delivered to myofibers. In addition, the fact that we have identified elements that confine expression to either fast- or slow-twitch fibers is extremely pertinent to gene therapy, since autosomal mutations underlie diseases like Duchenne's Muscular Dystrophy which predominantly affect fast muscle.

Proposed Course:

The focus of the work for the next year will be to identify the transcription factors that confine the expression of the TnI genes, as well as a battery of other genes encoding fiber-types-specific proteins, to specific muscles in response to motoneuron activity. In addition, we will continue to use cell culture and transgenic mice to analyze the SURE and the FIRE for cis-elements that are necessary, and maybe sufficient, to direct fiber-type-specific transcription. Dr. Calvo has begun experiments to test the effects of stimulation frequencies on muscles of our different transgenic mouse lines using telemetry. This methodology, based on the use of magnetic waves to generate currents from implanted coils, has the advantage that it will facilitate the stimulation of muscles in the living mouse.

Different approaches will be taken to isolate novel transcription factors, including the screening of expression libraries using the SURE and FIRE sequences as probes, differential display, and a single hybrid system in yeast. These complementary approaches utilize either function or the binding specificity of different proteins to isolate novel factors. Dr. Stauffer has begun to establish the binding conditions for different factors binding the TnI SURE. The isolated cDNAs will be tested for their expression patterns during muscle specification, their regulation by distinct stimulation patterns, and their capacity to activate transcription from the TnI slow and fast enhancers. Misexpression experiments in transgenic mice, and the inactivation of the genes by gene knock-out will be used to assess the functional properties of the factors during muscle specification and in the adult.

Protocols

Animals

93-030	Buonanno	Production of transgenic mice

Publications:

Calvo S, Stauffer J, Nakayama M, Buonanno A. Transcriptional control of muscle plasticity: differential regulation of troponin I genes by electrical activity. Dev Genetics, in press.

Gibney G, Buonanno A. Analysis of neural-responsive myogenin upstream sequences by myoblast implantation. Dev Biol 1995;172: 614-24.

Nakayama M, Stauffer J, Basu S, Wawrousek E, Buonanno A. Common core elements are found in skeletal muscle slow and fast fiber-type specific enhancers. Mol Cell Biol 1996;16:2408-17.

Rosenthal, N. and Buonanno, A. (1996) Molecular mechanisms regulating muscle plasticity. Dev Genetics, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00712-05 LDN

PERIOD COVERED

Regulation of Phenotypic Differentiation in the Developing Mammalian CNS

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

October 1, 1995 through September 30, 1996

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. v. Agoston	Visiting Scientist	LDN, NICHD
Others:	A. Dobi	Visiting Associate	LDN, NICHD
	F. Lim	Visiting Fellow	LDN NICHD
	C. Palkovits	Professional Services	LDN, NICHD
	M. Ring	Professional Services	LDN, NICHD
	A. Eitel	Research Assistant	LDN, NICHD

COOPERATING UNITS *(if any)*

LCB,NIMH (M. Palkovits); LN, NINDS (B. Andrews), NCRR, BEIP (R. Leapman); LMB, NINDS (R. McKay), NIDDK (B. Sauer), LMG, NIMH (U. Hochgeschwender); Dept. Neurology, USUHS (A. Verma); LDN, NINDS (L. Hudson), Dept. Neuroscience, UMDNJ (P. Levitt)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Unit on Molecular Control of Neurodifferentiation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.8	2.3	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The developmental regulation of phenotypic diversity in the mammalian central nervous system (CNS) was studied using the enkephalin phenotype as a model at the molecular and cellular level with special emphasis: a) on the identification and function of cis- and trans-acting elements of the rat enkephalin (ENK) gene; b) on understanding the involvement of DNA- and nuclear structure in this regulation; and c) on identification of cellular interactions and extracellular signals that induce phenotypic differentiation. The three components of the putative early (E12-E16) phenotypic specifier complex of the ENK gene (septamer-binding complex) were identified and characterized; the cDNA of the 43 kD member (ABP-43) of the late repressor complex (E21-P2) of the ENK gene that binds to the motif (ATT)19 encodes a novel nuclear protein; the whole ATT binding protein complex was translocated from the soluble nuclear protein fraction to the nuclear matrix fraction in a development-specific manner; the predicted development- and cell-specific repressor function of this ATT-protein complex was verified *in vivo* using various DNA molecular decoys in primary differentiating neurons; a caudate nucleus specific protein (CSP) that binds to a novel activator motif of the ENK gene was identified; this protein specifically binds to intranuclear actin that can act as molecular chaperone for CSP. Genomic DNA was found to bind calcium with high affininty (DNA>calmodulin or BAPTA) and induce specific structural changes depending on the DNA's superhelical density; differentially phosphorylated intranuclear proteins were competing with the DNA for calcium binding. The enkephalin phenotype was selectively regulated by specific cell-cell interactions that mimiced major basal ganglia connectivities. A new method (*in situ* DNA binding assay-ISDBA) was developed that enables spatial analysis of specific protein-DNA interactions. Intranuclear calcium was quantified in differentiating neurons using X-ray-based element microanalysis at the EM level that revealed "calcium hot spots" exclusive to heterochromatin. Myelin gene expression was found to be induced and regulated by electrical / neuronal activity in a frequency dependent manner in the differentiating CNS. Astroglia derived from the Down syndrome/Alzheimer model TS16 was found to depress cholinergic functions. ENK gene regulation in neonatal brain injury model was identified as age-dependent de-repression through the (ATT) repeat. The HIV repressor protein (TDP-43) of unknown normal cellular function interacts with the (ATT) repeat of the ENK gene in a development-specific manner.

Project Description:

Objectives:

1) To understand the developmental regulation of phenotypic diversity in the mammalian CNS.

2) To identify embryonic-specific cis-elements of the ENK gene and define their roles at the molecular and cellular level during phenotypic differentiation.

3) To identify, isolate and characterize their DNA binding proteins.

4) To investigate the role of far distant DNA-DNA interactions, structural changes of the DNA and the nuclear structure during neuronal differentiation.

5) To study the role of cellular interactions and the specificity of neuronal connectivity in the developmental induction of the enkephalin phenotype.

Methods employed:

PCR cloning and yeast-based two-hybrid system was used to isolate/clone various DNA-binding proteins. Mobility shift assays, competition-, supershift assays were used to identify and characterize embryonic- and brain-region-specific DNA-protein complexes. DNA binding was determined by using various wild type and mutant DNA probes. Various techniques were used to isolate nuclear proteins from our "developmental brain bank" and nuclear matrix associated proteins from freshly dissected embryonic brain regions. DNA footprinting techniques, including, methylation interference, DNAse I, osmium tetroxide footprinting were used to identify the specific cis-elements and to assess structural changes in the DNA. Biotinylated magnetic bead based assay system was used to study protein based far distant DNA-DNA interactions and for DNA affinity purification of nuclear proteins followed by microsequencing. Different protein purification methods including preparative isoelectrofocussing and gel fractionation were used to purify DNA binding proteins. Routine recombinant DNA techniques were employed to generate various transgenic constructs. Primary developing neuronal cultures as well as heterotopic and homeotypic co-cultures were from microdissected embryonic brains at different developmental stages. Proliferating multipotent neuroglial stem cell cultures which can be differentiated into glial and neuronal lineages were used. Various physical DNA transfer methods for transient transfection and DNA molecular decoy and competitive transfection assays were developed followed by either quantitative RT PCR of various marker mRNAs or various reporter gene activities were measured (CAT, B-gal, luciferase). In situ DNA binding assay was developed to localize specific DNA-protein interactions on a histological sections. Cell preparation techniques along with quick freezing techniques were developed to quantify and localize intranuclear calcium hot spots.

Major findings:

1. A set of embryonic intranuclear DNA binding proteins that form a development- and cell-specific complex with an alternative octamer [(A/Genk)TTTGCAT=sept] motif located at -542 relative to the transcriptional start site of the rat ENK gene were identified as putative phenotypic specifiers. These proteins form DNA-protein complexes of cell- and developmental stage specific manner between E12 and E18. The basic DNA binding protein unit (stem-sept) is a 34 kD dimer of two 17 kD phosphoproteins that are expressed as early as E12 in dividing neuroglial stem cells. The cell specificity of the complex is achieved by the expression of two additional proteins the 29 kD

neuron-specific or the 23 kD glia-specific proteins (neuro-sept and glia-sept respectively) that bind to the 34 kD core protein unit. The proteins (neuro-sept and glia-sept) can be used as highly specific markers of neuronal and glial precursor cells. All of these proteins can be differentially phosphorylated which can affect both the assembly of the protein complex itself and also the DNA binding activity. Differential phosphorylation can be regulated by extracellular signals which can affect phenotypic fate in the embryonic nervous system. These proteins are novel proteins however with some functional resemblance to known-octamer-binding proteins that are shown to specify cellular fate. Based on the available data the septamer motif and its binding proteins may serve as phenotypic specifiers at the early stage of differentiation which restrict the normal expression of the ENK gene to a subset of neurons. At the structural level the assembly of the various sept-complexes followed by the binding of sept-proteins to the DNA results in a characteristic DNA bending of specific degrees.

2. The 43 kD and the 90 kD members (ABP-43 and ABP-90) of the main developmental repressor complex of ENK gene that binds to the motif (ATT)19 as well as to the proximal promoter region of the gene have been DNA affinity purified and microsequenced. The cDNA of ABP-43 encodes a novel proline rich nuclear protein.). Competitive mobility shift assays with various mutants of the ATT motif indicated that the protein complex requires a specific DNA structure characterized by AT rich sequences characteristic to matrix attachment regions (MARs). This hypothesis was supported by in vivo experiments using the drug distamycin that specifically binds to the minor groove and thereby perturbs minor groove-protein interactions typical of nuclear matrix proteins.

The formation of this protein-DNA complex is restricted to a developmental window period when the cell-specific repression takes place, but within this period the abundance of the (ATT) binding complex showed a perfect negative correlation with the level of ENK expression. The formation of the protein-(ATT) complex was weakened or eliminated by distamycin treatment as observed in gel shift assays *in vitro* and resulted in a several fold increase in ENK mRNA levels when primary developing cortical neurons were treated with distamycin. During this period the (ATT)- binding proteins are abundantly present in the soluble nuclear fraction of future non-enkephalinergic neurons but found in the nuclear matrix fraction in adult neurons.

The presumed repressor role for the (ATT) complex was verified by DNA molecular decoy in primary differentiating neurons. Cortical, striatal and thalamic neurons that express high, medium and low or no ENK mRNA were decoyed with ds(ATT)19 DNA or control DNA for 48-72 hr. during the decisive early postnatal period (E21-P2). Decoying the protein complex resulted in a several fold increase in mRNA ENK but the extent of increase was different in the different brain regions showing the highest increase in the non-enkephalinergic thalamus. Similar results were obtained when the decoy DNA was co-transfected with reporter constructs that contained the (ATT) repeat as reporter gene activity increased several fold in (ATT) decoyed cultures.

These findings firmly support the originally proposed repressor role for the complex and for the first time indicate the involvement of the nuclear matrix and raise the possibility for development-specific intranuclear translocation of DNA binding proteins between various subnuclear compartments.

We have located the caudate-specific enhancer/activator element located at -1480 bp of the rENK gene. This element is a putative activator and binds a caudate nucleus specific protein: CSP). Intranuclear actin binds to CSP and has a seemingly molecular chaperone type of interaction with CSP.

3. The major calcium pool in the (neuronal) nucleus is associated with the DNA as DNA binds calcium with higher affinity than BAPTA. The DNA-calcium binding depends on the DNA's superhelical density as linearized DNA lacks DNA binding capacity. Several newly identified intranuclear calcium binding proteins, depending on their phosphorylated status that alters their calcium binding capacity, can compete with the DNA for calcium binding. New *in vivo* evidence supports our previously proposed model that TG/AC repeat sequences of the genome may be calcium responsive DNA-structural elements. X-ray based micro element analysis at the EM level showed large differences in subnuclear calcium distribution which is unique to this element, all other ions distributed through the nucleus evenly. The found significant variances in the subnuclear total calcium distribution points toward the existence of subnuclear "calcium hot spots" exclusively associated with heterochromatin. The measured calcium concentration values allow the structural response in these subnuclear "calcium hot spots". *In vivo* footprinting of the rENK gene genomic DNA indicates that the opened or closed state of the repeat corresponds to the functional status of the gene (activated vs. silenced). Based on the measured *in vivo* values, we modeled *in vitro* the calcium response of the TG/AC repeat. We showed that calcium and only calcium induces sliding and strand opening on TG/AC dinucleotide repeat. From these and other confirming evidences we are proposing a double calcium-pool model for calcium dynamism within the neuronal cell nucleus where genomic DNA and nuclear proteins compete for the calcium that upon binding to either of these components causes change in the molecular structure of the nucleus.

Significance to Biomedical Research and the Program of the Institute:

As more and more evidence points toward the developmental origin of major neuropsychiatric disorders, it has become imperative to understand the fundamental developmental processes that lead to the normal, balanced phenotypic diversity of the adult CNS. Basal ganglia especially has been the target of intensive research as a wide array of techniques - from molecular genetics to PET scan - demonstrated that it is the primary "locus morbus" of many mental diseases (autism, schizophrenia, Huntington's and Parkinson's diseases, etc.). The main peptide neurotransmitter of the basal ganglia circuit is enkephalin and its involvement in mediating pathological conditions such as autistic behavior, hallucinations in schizophrenia, personality changes in Huntington's disease, etc. has become more and more evident. Enkephalin, however, is not only a peptide neurotransmitter but also acts - along with other neuropeptides like VIP - as a critical extracellular signaling molecule during development and disease. In our model of neonatal brain damage, postnatal thalamic neurons that normally do not express enkephalin become temporarily "enkephalinergic" and the released enkephalin acts as a chemoattractant to direct immune cells to the venue of the damage and trigger their scavenger functions. Our research not only provides critical information about enkephalinergic gene regulation at many levels (from DNA structure to cell-cell interactions), but helps to understand the fundamental process of phenotypic differentiation in the developing CNS. Indeed, the emerging "big picture" from our studies is that the primary regulatory mechanism that controls gene expression in the developing nervous system is gradual and selective repression rather than specific activation. This repression is modulated by extracellular signals that specify the phenotype. However, once it has received the specifying signal, the differentiating neuron "spends" most of its time and energy to execute a whole array of molecular processes that repress the large majority of genes and thereby sculpture it s phenotype.

As "timing" seems to be one of the "master controllers" during neurodifferentiation, markers that indicate developmental stages can be of great use. Our approach ("sample and probe") has already identified a significant number of novel, development-specific DNA binding proteins and their functions, and these can also be used as markers that precisely indicate the stage of differentiation in the developing mammalian central nervous system.

Proposed Course:

Our highest priority is to understand the *in vivo* role for the identified DNA motifs individually and in combination during the various stages of phenotypic differentiation, as well as to characterize their specific DNA binding proteins and define their functions *in vitro*.

In vivo studies: We will continue to study the *in vivo* role of these motifs in both transgenic models and in primary developing neuronal cultures using transient transfection combined with DNA molecular decoy, as well as antisense oligonucleotide treatments followed by RT-PCR quantification of selected mRNAs.

a) Transgenic studies

We will determine cis-requirements for correct development-, cell- and brain region- specific expression of the ENK gene using the cre-lox targeted insertion system. Our first construct (pMENK/B-gal/lox5.0) has been made utilizing Brian Sauer's CRE-lox vector (pBS392). The basal and induced expressions of the reporter gene under control of the 5 kb 5' regulatory region of the ENK gene were successfully tested in a cell culture model. In the likely case of correct expression pattern in pMENK/B-gal/lox5.0 animals, in the next generation of transgenes we will attempt to perturb the developmental- and cell-specific expression pattern of the ENK gene by mutating all three elements of the septamer motif, the (ATT)19 repeat and [(TG/AC)28. The stem cells have been generated in Dr. Sauer's lab.

b) Primary neuronal culture project:

Parallel with the transgenic effort, we will continue to perform both transient transfection assays as well as DNA molecular decoy and antisense oligonucleotide treatments in various primary neuronal culture systems and constructs identical to those used for transgenes. In collaboration with Dr. Mackay's laboratory, we plan to use his neuralgia stem cell culture system as a cell culture model for both transient transfection as well as a DNA decoy to elucidate the function of the septamer motif. Also, to identify the nature of the inducing signal that triggers the specification of neurons into the enkephalinergic phenotype, we will continue the hetoro- and homeotypic co-culture systems combined with pharmacological treatments to mimic the effect of the specifier signal.

In vitro studies: We will isolate the additional members (ABP-90 and ABP-200) of the (ATT)19 repeat binding complex by using the two hybrid system. We have also started to use phage display for isolating the septamer binding proteins and the caudate-specific-binding protein (CSP). We have already made the libraries from E16 brain as well as P1 striatum/caudate nucleus. We will use the available sequence information to design antisense oligonucleotides for treating primary neuronal cultures and measure various mRNA by RT-PCR. Also, we will continue to locate the proximal regulatory region that, based on its closeness to the transcriptional start site, acts as an "effector" following its binding to the (ATT) complex and represses transcription.

Publications

Agoston Dv, Palkovits CG, Fitzgerald SF, Brenneman DE. Developmental changes in the inducibility of fos-like immunoreactivity in primary embryonic spinal cord cultures. Dev Brain Res 1995;89:173-86.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00713-02 LDN

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Gene Transcription and Neurite Outgrowth by Neural Impulse Activity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI	R.D. Fields	Head	LDN, NICHD
Others:	F. Eshete	Visiting Fellow	LDN, NICHD
	A. Lyckman	IRTA Fellow	LDN, NICHD
	B. Stevens	Biologist	LDN, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Unit on Neurocytology and Neurophysiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	1.8	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our studies show that different cell adhesion molecules (CAM) are regulated by different patterns of neural impulses, and that this has functional effects on key developmental processes in the nervous system. Expression of both calcium-dependent and calcium-independent classes of CAMs is regulated by neural impulse activity in DRG neurons, but different CAMs respond differently to different patterns of stimulation. Low-frequency stimulation down-regulates L1 mRNA and protein levels, but NCAM is not affected. Expression of N-cadherin is down-regulated by higher frequencies of stimulation than those affecting L1 expression. Studies *in vivo* show that levels of L1 mRNA decrease abruptly in DRG neurons at the embryonic period when spontaneous impulse activity begins, which is consistent with decreased expression of L1 following low-frequency stimulation in cultured DRG neurons. Activity-dependent regulation of L1 induces defasciculation of axon terminals, reduces adhesion and the mitotic rate of Schwann cells on axons stimulated at frequencies that lower L1 expression. Exposure to specific neurotrophins and appropriate target neurons from the spinal cord prevents the activity-dependent down-regulation of L1 in cultured DRG neurons. Studies of how intracellular signaling pathways in neurons integrate and transmit information coded in the temporal pattern of neural impulses support a mechanism in which the differing kinetics of parallel intracellular signaling reactions confers sensitivity to different temporal patterns of activation and thereby activates distinct intracellular pathways controlling gene expression and other neuronal and synaptic functions. These results are based on studies of the signaling pathways (second messengers, protein kinases, and transcription factors) that activate the immediate early gene *c-fos* in response to different patterns of action potential stimulation *in vitro*.

Project Description

Objectives

The Unit on Neurocytology and Physiology, directed by **Douglas Fields**, is investigating how expression of genes known to regulate proteins that are important in development of the nervous system (cell adhesion molecules, voltage-gated calcium channels, and immediate early genes), can be regulated by specific patterns of neuronal impulses in neurons, and thereby provide an important influence on the developing structure and function of the nervous system. Effort is now focused on two related areas: (1) identifying important biological consequences of activity-dependent regulation of these genes, and (2) identifying the signaling mechanisms responsible for regulating gene expression by specific patterns of membrane depolarization.

Methods Employed

Dorsal root ganglion neurons dissociated from fetal mouse spinal cord are maintained in a multi-compartment cell culture preparation equipped with platinum electrodes for chronic electrical stimulation. Other cell culture preparations include spinal cord neurons, Schwann cells dissociated from the sciatic nerve of mice and neuroblastoma cells.

Electrophysiological recording, advanced imaging techniques, and molecular methods are employed to study neuronal responses to action potential stimulation. Growth cone responses to guidance factors and action potentials are studied by low-light level time-lapse video microscopy. Ratiometric imaging of calcium-sensitive dyes is used to measure the temporal and spatial dynamics of electrically-evoked calcium transients in neurons. Immunocytochemistry, electron microscopy and confocal microscopy are used for morphological studies.

Specific mRNA transcripts in cultured neurons are measured by polymerase chain reaction, competitive PCR, in situ hybridization, and Northern blots. Western blot, immunoprecipitation and kinase assays are used to monitor expression of neural cell adhesion molecules, immediate early genes, neurotrophin receptors, CaM Kinase and MAP kinase activity, and phosphorylation of the transcription factors SRF and CREB.

Surgical implantation of miniperfusion pumps to infuse protease inhibitors into the brain are being used in combination with trans-synaptic neuronal labeling to investigate the formation of ocular dominance columns in visual cortex of kittens.

Major Findings

Regulation of Neural Cell Adhesion Molecules by Specific Patterns of Neural Impulses. Specific cell adhesion molecules (CAMs), expressed selectively on different neurons, regulate many of the major processes in development of the nervous system, including cell recognition, aggregation, motility, neurite outgrowth fasciculation and synaptogenesis. Although the expression of distinct neural cell adhesion molecules is regulated precisely during development of the nervous system, our research has shown that expression of the cell adhesion molecule L1 is also controlled by electrical impulse activity in mouse DRG neurons in vitro. Correlation of stimulus patterns studied in vitro with activity patterns in utero suggest that this mechanism could help coordinate the developing structure and function of the nervous system.

In the past year we have found that different CAMs are regulated by different patterns of neural impulses. In DRG neurons, NCAM is not regulated at the mRNA or protein level by frequencies of impulses that affect L1 expression, but a member of the calcium-dependent class of CAMs, N-cadherin, is down regulated by impulse activity of higher frequencies than those that will affect L1 expression. The time-course of activity-dependent regulation has been determined *in vitro* and *in vivo* for the L1 gene, and we have found that L1 returns to normal levels after terminating impulse activity in cell culture. Levels of L1 mRNA increase between embryonic day 11 (E-11) and E-14 in mouse DRG, but levels decrease sharply after E-15. This decreased expression coincides with the time when spontaneous impulse activity, similar to that which lowers L1 expression in culture, begins in these neurons *in vivo*.

We have determined that there are significant functional consequences of activity-dependent regulation of the L1 gene that could affect nervous system development through effects on cell-cell adhesion and axonal fasciculation. Involvement of L1 in axonal-glial association has led us to investigate the possibility that neural impulse activity could regulate neuronal-glial interactions dependent on L1. Brd-U labeling of mitotically active Schwann cells in co-cultures of DRG neurons shows that proliferation of Schwann cells is reduced on axons stimulated at a frequency that lowers L1 expression. Axon myelination is an important developmental process relevant to nervous system disease, and we have developed an in vitro assay of axon myelination and used biochemical markers (Gal-C and MAG) and morphological criteria to investigate differences in Schwann cell myelination of DRG axons stimulated to fire action potentials at different patterns in culture.

Other research has shown important interactions between neurotrophins, electrical activity, and cell adhesion molecules. We have determined that specific neurotrophins can prevent the activity-dependent regulation of L1, and we are using specific inhibitors of the Trk receptors to determine which receptors and pathways are involved. Initial studies indicate that the effects of neural impulse activity vary depending upon whether the neuron has or has not innervated appropriate target structures. For instance, L1 expression is not influenced by electrical activity in DRG neurons that have innervated appropriate neurons in the ventral spinal cord. Present evidence suggests that synaptogenesis with the target is not required for this inhibition, but rather that this regulation is mediated by a diffusible signaling molecule secreted by target neurons.

Intracellular Signaling from Neural Impulses. We are monitoring the temporal dynamics of key elements (second messengers, protein kinases, transcription factors) that regulate expression of the immediate early gene *c-fos* in response to membrane depolarization in an effort to understand how intracellular signaling pathways integrate and transmit information coded in the temporal pattern of neural impulses. Results of these studies are consistent with the idea that the differing kinetics of parallel intracellular signaling reactions confers sensitivity to different temporal patterns of activation and thereby activates distinct intracellular pathways controlling gene expression or other neuronal and synaptic functions. We find that transcription of the immediate early gene *c-fos* is sensitive to the pattern of action potential stimulation, and correlated inversely with the interval between successive bursts of action potentials when stimulus bursts are separated by intervals of a few minutes. We do not find that the concentration of any one element in the signaling cascade accounts for the transcriptional regulation in response to different frequencies of activation; instead, the time-dependent concentration dynamics of different elements in the cascade contribute to the transcriptional response to different frequencies of stimulation. For example, the interval between calcium transients correlates with *c-fos* expression, but the amplitude of the calcium transient does not. Phosphorylation of the transcription factor CREB at serine 133 parallels the rapid rate of increase in $[Ca^{++}]_i$, but $[Ca^{++}]_i$ recovers to basal levels much faster than CREB dephosphorylation. For some temporal patterns of stimulation, phosphorylation of CREB at serine 133 was not well correlated with *c-fos* expression. Although CREB is a key regulator of *c-fos* transcription, the results suggest that CREB phosphorylation at serine 133 need not be the determining factor in response to certain patterns of neural impulses.

Significance to Biomedical Research and the Program of the Institute

Nervous system development and recovery from nervous system injury are critically dependent upon selective cell-cell adhesion, fasciculation, and growth cone guidance. The regulation of these and other developmental processes by extracellular signals is essential for coordinating the complex structural/functional interrelations necessary for development and regeneration of the nervous system. Elucidating the responses of signaling reactions that link action potential stimulation to gene transcription, and that in turn activate specific adaptive responses in neurons, will ultimately enable better interventions to prevent or correct developmental deficits and promote recovery from nervous system injury.

Proposed Course

Future work will emphasize the involvement of cell adhesion molecules in activity-dependent plasticity, and interactions between neurotrophins, cell adhesion molecules and electrical activity. Functional consequences of activity-dependent regulation of cell adhesion molecules will be explored with emphasis on neuronal-glial interactions, and axon fasciculation. Mechanisms of growth cone outgrowth and guidance in response to diffusible and substrate bound factors will be studied in neuronal culture. Molecular mechanisms of long-term depression and relation to synapse elimination will be investigated in visual cortical neurons in culture and in vivo.

Protocols:

Animals:

96-015	Fields	Activity-dependent nervous system development
94-016	Fields	Effects of proteases on ocular dominance columns of visual cortex

Publications

Fields RD, Itoh K. Neural cell adhesion molecules in activity-dependent development and synaptic plasticity. Trends Neurosci, in press.

Fields RD Signaling from neural impulses to genes. The Neuroscientist, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
ZO1 HD 00714-01 LDN

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular mechanisms of synapse development and plasticity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Bai Lu	Unit Head	LDN,NICHD
Others:	Alexander Figurov	Visiting Fellow	LDN,NICHD
	Keiko Mizuno	Visiting Associate	LDN,NICHD
	Chikara Oho	Visiting Fellow	LDN,NICHD
	Xiang-ping He	Technician	LDN,NICHD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Developmental Neurobiology

SECTION
Unit on Synapse Development and Plasticity

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The long-term goal of this laboratory is to understand the molecular mechanisms underlying synapse development and function. A number of model systems are used in our studies, including Xenopus nerve-muscle cultures, dissociated cultures of mammalian neurons, hippocampal slices and knockout mice. Our recent efforts focus on two closely related areas, using a combination of molecular biological and electrophysiological techniques: 1) to study the role of neurotrophic factors in synapse development and plasticity; 2) to isolate new genes and characterize new molecules that are involved in specific stages of synaptogenesis. We have discovered that the neurotrophin BDNF promotes the development of long-term potentiation, a cellular model for learning and memory. This effect is mediated by a potentiation of synaptic responses to high frequency stimulation. These results suggest that neurotrophins regulate synaptic maturation. We also demonstrated that endogenous synapsin I, a synaptic vesicle protein that is a target of neurotrophic regulation, is an important molecule mediating synapse maturation. Finally, we cloned the mammalian homolog of frequenin, a calcium binding protein involved in synaptic responses to high frequency stimulation.

Project Description:

Objective: To study the role of neurotrophic factors in synapse development and plasticity; and to isolate and characterize new molecules that are involved in specific stages of synaptogenesis.

Major Findings.

1. Neurotrophic regulation of the development of long-term potentiation.

Neurotrophic factors are traditionally viewed as factors that regulate neuronal survival and differentiation. An exciting new era of research on the role of neurotrophic factors in synapse development and plasticity has recently emerged. Last year, we reported that BDNF and NT3, which belong to the neurotrophin family of proteins, promote the maturation of neuromuscular synapses. We also extended the work into central nervous system (CNS) and showed that these neurotrophins have the capacity to rapidly modulate neurotransmitter release. To determine the physiological significance of neurotrophic modulation of neurotransmitter release in the brain, particularly with regard to synapse development, we studied the effect of BDNF on long-term potentiation (LTP) in the hippocampus. LTP is believed to be a cellular mechanism for learning and memory. Study of cellular and molecular mechanism for LTP is one of the hottest research topics in the modern neuroscience because of its significant implications in the understanding of brain function and dysfunction. In developing hippocampus, the ability to undergo LTP induced by tetanic stimulation increases with the age, paralleling the expression of BDNF and its receptor TrkB. Exogenous BDNF promoted the induction of LTP in young (postnatal day 12-13) hippocampal slices, which in the absence of BDNF show only short-term potentiation. In contrast, a TrkB-IgG fusion protein, which scavenges endogenous BDNF, reduced the magnitude of LTP in adult hippocampus. Consistent with studies using BDNF knockout mice, a critical level of BDNF/TrkB activity appears to be important for long-term modulation of synaptic efficacy. Exogenous BDNF promoted synaptic responses to tetanus and enhanced LTP in neonatal hippocampus where endogenous BDNF level is low, while TrkB-IgG had opposite effects in adult hippocampus where endogenous BDNF level is high. The effect of BDNF on LTP development was due to an activation of TrkB receptors, because it can be blocked by TrkB-IgG, and NT4/5 which activates the same TrkB receptor had a similar effect as BDNF. We further examined the mechanism underlying BDNF regulation of LTP. We found that BDNF had no effect on basal synaptic transmission but increased the synaptic responses to high frequency stimulation in P12-13 slices, while TrkB-IgG had the opposite effects in adult slices. We also found that LTP induced by pairing low frequency stimulation with postsynaptic depolarization was not affected by BDNF or TrkB-IgG. Thus, the effect of BDNF on LTP development was due to an enhanced ability of hippocampal synapses to respond to tetanic stimulation, rather than to a direct modulation of LTP-triggering mechanism. Our results suggest that BDNF may play an important role in long-term synaptic plasticity such as LTP, and therefore may provide insights into molecular mechanisms for the activity-dependent regulation of synaptogenesis and plasticity.

2. Synapsin I as a molecular mediator for synaptic maturation.

Our previous experiments suggest that the synaptic vesicle associated protein synapsin I is involved in synapse maturation. Thus, loading of purified, exogenous synapsin I into Xenopus embryos promoted physiological as well as morphological maturation of neuromuscular synapses in culture. Treatment of the Xenopus nerve-muscle culture with the neurotrophins BDNF and NT3 resulted in an earlier maturation of neuromuscular synapses based on morphological and physiological criteria, and this maturation is accompanied by an increase in the cellular levels of synapsin I in the motor neurons. We now provide evidence that endogenous synapsin I may be responsible for the maturation of neuromuscular junction. First, we examined the expression of Xenopus synapsin I (XsynI) with a specific anti-XsynI antibody both *in vivo* and in culture, in comparison with that of synaptophysin, another synaptic vesicle protein. We found that the expression of XsynI was much later than that of synaptophysin, and correlated well with synapse maturation, rather that initial synaptic formation. Second, we developed a way to induce an earlier expression of endogenous XsynI, by treating the culture with laminin, a cell adhesion molecule known to be involved in synapse development. We found a dramatic induction of XsynI expression by laminin. Electrophysiological recording indicated that all synapses that have high levels of endogenous synapsin I exhibited mature properties. Thus, functionally maturation of synapses may depend upon the expression of XsynI at the presynaptic terminals

3. Cloning and characterization of mammalian calcium binding protein frequenin.

Another major effort in the lab is to isolate and characterize new molecules that are involved in specific stages of synaptogenesis. In the past few years we have identified the following molecules that play a role in different stages of synaptogenesis:

1) Synapsin I is an important presynaptic molecule for synapse maturation.
2) CGRP potentiates synaptic responses at developing neuromuscular synapse.
3) Nitric oxide as a retrograde signal for synaptic depression.
4) Cloning and characterization of frequenin, a Ca2+-binding protein potentially involved in synaptic development and plasticity.

We have previously shown that frequenin facilitates neurotransmitter release at Xenopus neuromuscular synapses. To determine the cellular and subcellular localization of the protein and therefore provide some clues as to how frequenin achieve its function, we cloned mouse frequenin (Mfreq) and characterized its expression both *in vivo* and in culture. The amino acid sequence of Mfreq is homologous to that of frequenins from other species. Northern and Western blot analyses indicated that the Mfreq mRNA is a single species of 4.2 kDa, and that the protein has a mass of 24 kDa protein on SDS gel, respectively. Expression of Mfreq is nervous system specific. However, Mfreq mRNA and protein are widely distributed in the brain, spinal cord, and dorsal root ganglia. Mfreq is expressed in early embryonic brain and the levels of Mfreq remain high throughout development. *In situ* hybridization and immunocytochemistry demonstrated that Mfreq is expressed primarily in neurons and presumptive astrocytes. The Mfreq protein was preferentially localized in neurites (dendrites and axons). Double immunofluorescence microscopy established that Mfreq was co-localized with the dendritic marker, MAP-2 and the synapse marker, SV2 in cultured hippocampal neurons. The distribution and subcellular localization of Mfreq may help understand its cellular function.

Proposed Course of Project:

1. Mechanisms underlying BDNF regulation of LTP development. We have shown that BDNF promotes LTP development by enhancing synaptic responses to high frequency, tetanic stimulation. How BDNF regulates synaptic responses to tetanus is a question important not only for studying BDNF action in the hippocampus but also for understanding the basic mechanism of synaptic transmission and plasticity. First, we need to distinguish whether BDNF acts either presynaptically to prevent a run down of neurotransmitters; or postsynaptically to attenuate the desensitization of glutamate receptors. Our preliminary studies on paired pulse facilitation and synaptic depression suggest that at least there is a presynaptic component. We will try to localize TrkB receptors on either presynaptic nerve terminals or postsynaptic dendritic spines, using immuno-electron microscopy. Drugs that block the desensitization of glutamate receptors

will also be used to see if they prevent BDNF effects. Second, signal transduction mechanisms will be studied. There are at least three pathways that are postulated to mediate neurotrophin signaling: the MAP kinase pathway; the PI3 kinase pathway; and the less well characterized SNT transcription factor pathway. High frequency response in the hippocampus may serve as a convenient assay to study the signaling mechanism for the synaptic effect of BDNF, which is completely unknown at present. Pharmacological approaches will be used to inhibit a specific pathway and see if the BDNF effect is blocked. The activities of specific kinases involved in individual pathways will be measured in slices treated with BDNF to determine which pathway is activated under our experimental conditions. Third, we would like to identify molecular target of BDNF actions. Using BDNF knockout mice as well as cultures or slices treated with or without BDNF, we will examine the expression of a variety of synaptic markers, particularly synaptic vesicle (SV) proteins potentially involved in high frequency synaptic responses. Two prime candidates are synapsin I and synaptotagmin, because injection of antibodies to these proteins into nerve terminals has been shown to cause synaptic depression in response to high frequency stimulation. Our preliminary experiments indicated that BDNF increased the expression of synapsin I and synaptophysin in neuronal cultures. Moreover, BDNF knockout mice exhibited no LTP and much pronounced synaptic depression after tetanus, and these phenotypes can be rescued after treatment of the slices with BDNF. It is possible that BDNF facilitates transmitter release at high frequency by up-regulating the expression and/or phosphorylation of certain SV proteins. Another candidate will be frequenin, which modulate synaptic responses to high frequency stimulation. Finally, using rapid freezing/electron microscopic techniques, we will determine which step(s) in the synaptic vesicle cycle BDNF exerts its effect. The synaptic vesicle cycle includes: transport of vesicles to the terminals; docking of vesicles to the presynaptic active zone; fusion of vesicles to the presynaptic membranes; and endocytosis and formation of new vesicles. Facilitation on any of these steps by BDNF could lead to an enhanced response to tetanus. By freezing the control and BDNF treated slices during the course of tetanic stimulation, we will see the morphological changes at the nerve terminal that will indicate the specific step that BDNF affects.

2. Neurotrophins as retrograde messengers for synaptic potentiation.

Retrograde messengers have long been thought to mediate activity-dependent modulation of synaptic efficacy. However, so far there is no definitive proof of any molecules as the retrograde messengers. The neuromuscular junction offers a simple model to study retrograde messenger because of the easily identifiable pre- and postsynaptic components. At the neuromuscular synapses, the neurotrophins BDNF and NT3 are synthesized in the postsynaptic muscle cells while TrkB receptors are expressed on presynaptic terminals. An ongoing project in the lab is to test whether NT3 may serve as a retrograde messenger for activity-dependent enhancement of neuromuscular synapses during early development. We found that membrane depolarization, elicited either by depolarizing agents or repetitive electric stimulation, rapidly and specifically increased the levels of NT3 mRNA in developing Xenopus muscle cells in culture. NT3 gene expression was also enhanced by ACh, the neurotransmitter that causes muscle membrane depolarization. The effects of depolarization were mediated by increasing intracellular calcium concentration. Moreover, the depolarization-induced NT3 potentiated synaptic activity at the developing neuromuscular synapses. The frequency of spontaneous synaptic currents recorded from neuromuscular synapses was markedly increased after treatment with conditioned medium from depolarized muscle cultures. This effect of conditioned medium was partially blocked by TrkC-IgG fusion protein, a specific scavenger of NT3, suggesting that at least some of the released materials is indeed NT3. A number of experiments will be performed to further the retrograde role of NT3. We will confirm the presynaptic nature of NT3 action. We will further investigate the long-term effect of the trophin on synaptic maturation, electrophysiologically, biochemically and morphologically. These experiments will establish that muscle-derived NT3 may serve as a retrograde message for activity-dependent synaptic strengthening at the developing neuromuscular junction.

Significance to Biomedical Research and the Program of the Institute:

Neurotrophic factors are important biological regulatory mechanisms for the development and normal function of the nervous system. Studies on neurotrophic regulation of synaptic function is an exciting new field of neuroscience research and bears important implications in both synapse development and learning and memory. Moreover, identification of factors regulating synaptic efficacy may also lead to development of therapeutical agents for neurological disorders in both children and adults.

Publications:

Figourov A, Pozzo Miller L, Wang T, Olafsson P, Lu B. Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 1996;381: 706-9.

Lu B, Papov S, Czernik A, Wang T, Poo M-M, Greengard P. Expression of synapsin I reflects maturation of neuromuscular synapse, Neurosci, in press.

Olafsson P, Soares HD, Wang T, Herzog K-H, Morgan JI, Lu B. The Ca^{2+} binding protein frequenin is a nervous system-specific protein preferentially localized in neurites. Mol Brain Res, in press.

LEGR

NICHD Annual Report
October 1, 1995 to September 30, 1996

Laboratory of Eukaryotic Gene Regulation

GENERAL AMINO ACID CONTROL IN YEAST

Yeast cells respond to starvation for amino acids or purines by the global activation of genes encoding enzymes involved in amino acid and purine biosynthesis; this regulatory mechanism is termed general amino acid control. ***Alan Hinnebusch*** and his colleagues have elucidated the molecular mechanisms responsible for several aspects of this general control response. They have shown that the major "choke-point" in the system is translational induction of GCN4, a transcriptional activator of the co-regulated biosynthetic genes. Genetic and molecular studies of this translational mechanism have elucidated several steps in the translation initiation process involved in the binding of initiator tRNA to the ribosome. The analysis of mutations affecting *GCN4* translation has identified many of the subunits of translation initiation factors eIF2, eIF2B, and eIF3, as well as novel factors involved in the biogenesis of initiator tRNA. Furthermore, it has provided molecular insights into the mechanism wherein phosphorylation of eIF2 down-regulates translation under stress or starvation conditions, and of different modes for regulating the eIF2α kinases. This group is also investigating the mechanism of transcriptional activation by GCN4, focusing on the fine-structure of the activation domain and its physical interactions with different components of the transcriptional machinery. Finally, they are probing the molecular basis for purine repression of adenine biosynthetic genes, and have shown that modulating direct interactions between a Myb-related DNA binding protein (BAS1) and a homeodomain transcriptional activator (BAS2), is a key aspect of adenine repression.

Translational control by phosphorylation of eIF2 and inhibition of the guanine nucleotide exchange factor eIF2B. *GCN4* expression is repressed at the translational level by four short upstream open-reading-frames (uORFs) in the mRNA leader. In cells starved for amino acids, ribosomes bypass the uORFs and translate *GCN4* instead, because they fail to bind the ternary complex consisting of eIF2, GTP and charged initiator $tRNA^{Met}$ until after scanning past uORF4. This occurs because the concentration of ternary complexes is reduced in starved cells by phosphorylation of the α subunit of eIF2 by the protein kinase GCN2. Previously, Hinnebusch and co-workers obtained several lines of evidence indicating that phosphorylation of eIF2 converts it from a substrate to an inhibitor of its own guanine nucleotide exchange factor, eIF2B. First, overexpression of the three subunits of eIF2 or all five subunits of eIF2B reversed the inhibition of growth and derepression of *GCN4* translation associated with eIF2 phosphorylation by hyperactivated forms of GCN2 or by the human eIF2α kinase PKR. Second, a large number of point mutations were isolated in the GCN3, GCD7, and GCD2 subunits of eIF2B that suppress the effects of eIF2 phosphorylation without affecting the catalytic activity of eIF2B. These three subunits are homologous in sequence and the regulatory mutations map in two ca. 70-amino acid clusters located in a similar location in each protein. In fact, some of the mutations affect identical amino acid positions in two or all three of the subunits. They have now obtained genetic and physical evidence that these mutations do not affect the stability of eIF2B, nor do they act by excluding GCN3, the only dispensable subunit of the eIF2B complex. They propose that GCD2, GCD7 and GCN3 comprise a regulatory subdomain in eIF2B and that homologous regulatory segments in the three proteins are juxtaposed on the surface of the complex where they contact residues in eIF2α surrounding the phosphorylation site. Because the majority of eIF2 is phosphorylated in several of the regulatory mutants without any detectable inhibition of growth, it appears that the mutant forms of eIF2B can catalyze exchange on phosphorylated eIF2 rather than being inhibited by it. A plasmid system for overexpression and affinity-purification of eIF2B complexes has been developed to allow biochemical analysis of the effects of key regulatory mutations on the efficiency of nucleotide exchange on the phosphorylated substrate.

Their model for eIF2B organization is also supported by the previous finding that overexpression of GCD2, GCD7 and GCN3 in vivo leads to formation of a stable eIF2B subcomplex that can be immunopurified from cell extracts. Overexpression of this subcomplex rescues native eIF2B from inhibition by phosphorylated eIF2, presumably by sequestering eIF2(αP). Overexpression of only GCD2 and GCD7 also rescues eIF2B from eIF2(αP) by titrating the GCN3 subunit from the native complex, leaving a 4-subunit form of eIF2B that is less resistant to the inhibitor. Genetic and biochemical evidence has been obtained recently indicating that GCD2 directly interacts with GCD7 and that these interactions are dependent on regions of sequence similarity shared between the two proteins. Thus, homologous regions in GCD2 and GCD7 are involved in subunit interactions with one another in addition to mediating interactions with eIF2α.

Identification of GCD10 as the RNA-binding subunit of eIF3. Mutations in *GCD10* have the same effects on *GCN4* expression and general translation as do mutations affecting subunits of eIF2 or eIF2B. Previously, Hinnebusch and colleagues showed that GCD10 is essential for translation initiation in vivo, and found that it co-purifies and co-immunoprecipitates with the PRT1 subunit of eIF3. They went onto demonstrate that GCD10 is identical to the 54 kDa RNA-binding subunit of yeast eIF3. In view of the biochemical properties of mammalian eIF3, they proposed that *gcd10* mutations derepress *GCN4* translation because they impair the binding of ternary complexes to 40S subunits. This hypothesis was consistent with the isolation of all four *IMT* genes encoding $tRNA_i^{Met}$ as dosage suppressors of *gcd10* mutations, as increased levels of $tRNA_i^{Met}$ could increase ternary complex abundance and compensate for decreased efficiency of ternary complex binding to ribosomes.

They have now found that steady-state levels of $tRNA_i^{Met}$ are reduced in *gcd10* mutants, probably due to increased turnover, and that high-copy *IMT* genes simply restore wild $tRNA_i^{Met}$ levels in the mutants. In fact, *GCD10* can be deleted in strains overexpressing $tRNA_i^{Met}$, indicating that accumulation of $tRNA_i^{Met}$ is the essential function of *GCD10*. The *GCD14* gene has recently been implicated in the same function and GCD10 and GCD14 were shown to co-immunoprecipitate and co-purify with one another. At present there is little evidence that GCD14 is associated with eIF3; in addition, substantial amounts of GCD10 and GCD14 have been localized to the nucleus by indirect immunofluorescence. They speculate that GCD10 is in a nuclear complex with GCD14 that functions in processing or transport of $tRNA_i^{Met}$, in addition to its role in the cytoplasm as a constituent of eIF3.

Using a polyhistidine-tagged form of PRT1, Hinnebusch and colleagues are carrying out affinity-purification of eIF3 to identify all of the components of this complex. This work has already led to identification of NIP1, a previously identified protein involved in nuclear import, as an ca. 110 kDa subunit of eIF3. In addition, they found that the p130, NIP1, PRT1 and p39 subunits constitute a core complex of eIF3 and that GCD10, SUI1, and an unknown 34 kDa protein are more loosely associated subunits. Affinity purification of a distinct GCD10-containing complex using His-tagged GCD10 is also underway.

Role of autophosphorylation and protein dimerization in activation of GCN2 kinase function. GCN2 contains several regulatory domains located N-terminal and C-terminal to its protein kinase (PK) catalytic domain, including a region related to histidyl-tRNA synthetase (HisRS) and a C-terminal ribosome-binding domain. The HisRS-related domain can bind tRNA in vitro and it is believed to function in vivo to stimulate the PK domain upon binding of uncharged tRNA in amino acid-starved cells. The C-terminal domain is also required for ribosome binding and functions by an undefined mechanism in kinase activation. Using the 2-hybrid technique, it was found that the protein kinase (PK), HisRS-related, and C-terminal ribosome binding domains in GCN2 can interact with themselves and that the kinase domain and C-terminal domains can also interact with one another. Dimerization of GCN2 in cell extracts has been confirmed by co-immunoprecipitation experiments and is also consistent with the identification of dominant-interfering *gcn2* alleles. They now propose that GCN2 functions as a dimer and that interactions between the PK and C-terminal domains are involved in regulating kinase activity by uncharged tRNA.

Several protein kinases are known to require phosphorylation on conserved Thr or Ser residues located between kinase subdomains VII and VIII for catalytic function. Hinnebusch and colleagues have found that Thr-813 and Thr-818 in GCN2 are autophosphorylated in vitro and that Thr or Ser residues at these positions are required for *GCN2* function in vivo. These results suggest that autophosphorylation by GCN2 on these Thr residues is an important step in GCN2 activation.

Evidence that GCN1 and GCN20 function on the ribosome as activators of the eIF2α kinase GCN2. It was shown previously that the GCN1 and GCN20 proteins are components of a high molecular weight complex required in vivo for phosphorylation of eIF2α by GCN2. An N-terminal segment of GCN20 and an internal segment of GCN1 that is closely related to translation elongation factor 3 (EF3) were found to mediate complex formation between the two proteins. GCN20 also contains a region highly similar to EF3 comprising two ATP-binding cassettes. By immunofluroescence experiments, they showed that GCN1 is distributed throughout the cytoplasm, and biochemical fractionation studies now indicate that both proteins bind to polysomes and translating 80S ribosomes. From these results, they propose that GCN1 and GCN20 function at the ribosomal A site to detect uncharged tRNA and mediate the activation of ribosome-bound GCN2. Ribosome-binding by GCN20 is largely dependent on GCN1, whereas GCN1 can bind in the absence of GCN20. Ribosome binding by both proteins is stimulated by ATP, and the effect of ATP on GCN20-binding is altered by mutations in the GCN20 ATP-binding cassettes. Thus, ATP may stimulate complex formation between GCN1 and GCN20. Human homologues of both GCN1 and GCN20 have been identified, suggesting that this signal transduction mechanism is conserved between yeast and humans.

Mechanism of transcriptional activation by GCN4. In addition to studying translational control of *GCN4,* Hinnebusch and colleagues have been working towards a detailed structural model of the GCN4 transcriptional activation domain, and the identification of components of the transcriptional machinery that directly interact with GCN4. Extensive mutational analysis has shown that GCN4 contains 7 subdomains, each consisting of 2-3 bulky hydrophobic amino acids surrounded by acidic residues, located throughout the N-terminal ca. 130 amino acids of the protein. These hydrophobic clusters can cooperate in different combinations to activate transcription in vivo, indicating extensive functional redundancy. Two of the 7 clusters at positions 97-98 and 120-124 are especially critical for activation, and one or the other must be present in combination with more N-terminally located clusters for significant activation in vivo. Point mutations in the hydrophobic clusters are being used to test the specificity of in vitro interactions between GCN4 and various transcription factors. Results thus far reveal specific interactions between GCN4 and the $TAF_{II}60$ and $TAF_{II}90$ components of TFIID, and also with several SRB components of the mediator complex of RNA Polymerase II holoenzyme, in whole cell yeast extracts. Experiments are underway with purified TFIID and holoenzyme preparations to determine whether these interactions with GCN4 are direct.

Transcriptional control of adenine biosynthetic genes. The mechanism for repressing the transcription of adenine biosynthetic genes by excess adenine is also being examined. Previously, an *ADE5,7* promoter fragment of 67 bp was shown to be sufficient to confer adenine-repressible transcription on a heterologous promoter. Saturation mutagenesis of this fragment revealed three positive-acting elements of 6-10nt in length, that were thought to function as binding sites for the BAS1, BAS2, and ABF1 activator proteins. This has now been confirmed by in vitro DNA-binding experiments with purified proteins or in whole cell extracts by showing that mutations in the critical elements which abolish activation in vivo also reduced DNA-binding by the corresponding proteins in vitro. From these results it was proposed that adenine repression involves down-regulating the functions of one or more of these activators proteins by a negative regulator that does not itself bind to DNA.

Hinnebusch and colleagues have used fusions between with the bacterial LexA DNA-binding domain with BAS1 and BAS2 to analyze the mechanism of adenine repression. They found that LexA-BAS1 activated transcription from a *lexA* operator in a manner that depended on BAS2 and was repressed by adenine in the medium, whereas LexA-BAS2 activated transcription independently of BAS1 and adenine levels. Deletion analysis of LexA-BAS1 indicated that BAS1 contains a potent activation domain that is masked by inhibitory segments at its N- and C-terminal ends, and showed that this activation domain is unmasked upon complex formation with BAS2. Based on the fact that overexpressing BAS2 allowed both LexA-BAS1 and authentic BAS1 to activate transcription more effectively under adenine-repressing conditions, they concluded that complex formation between BAS1 and BAS2 is down-regulated by adenine. Taken together, their results indicate that complex formation by BAS1 and BAS2 is crucial for generating a potent transcriptional activator at *ADE* gene promoters and is a key step in *ADE* gene transcription that is repressed by adenine. A point mutation in BAS1 has been isolated that appears to permit more efficient complex formation with BAS2 in the presence of excess adenine.

STRUCTURE AND FUNCTION OF eIF2 AND ITS REGULATION BY PROTEIN KINASES

The group headed by ***Thomas Dever*** is seeking to identify the structural determinants of substrate recognition by the eIF2α kinases, and to illuminate the cellular and viral mechanisms employed to control eIF2 function by modulating the functions of these kinases. eIF2 plays a pivotal role in the initiation of protein synthesis by delivering initiator $tRNA^{Met}$ to the ribosome, and also functions in recognition of the AUG start codon during the scanning process. Therefore, it probably interacts with many other initiation factors and with the ribosomal acceptor site during the course of initiation. Inhibition of eIF2 by phosphorylation of its α subunit is a highly conserved mechanism for reducing the rate of protein synthesis under conditions of starvation and stress in yeast and mammalian cells. In addition, it mediates gene-specific translational induction of *GCN4* expression in yeast. The human eIF2α kinase PKR has growth suppressive properties and its induction is an important aspect of the interferon-mediated antiviral response. In addition, PKR has been implicated as a tumor suppressor in mammalian cells. It is appropriate, therefore, to exploit the powerful tools of yeast molecular genetics to probe all aspects of the structure, function and regulation of eIF2, its protein kinases, and regulators of these kinases found in mammalian cells. In addition to their studies on eIF2 and its kinases, this group is exploring a yeast protein homologous to bacterial initiation factor 2 to determine whether it functions in cap-independent internal initiation events.

Molecular determinants of substrate recognition by eIF2α kinases identified using a pseudosubstrate inhibitor. To identify amino acids in eIF2α that are critically required for its recognition by eIF2α kinases, Dever and colleagues have been carrying out a mutational analysis of the K3L protein, a pseudosubstrate inhibitor of PKR encoded by vaccinia virus. They established a system for suppressing the growth inhibitory effects of PKR in yeast cells by coexpressing K3L protein, and using this system identified both loss-of-function and hyperactive K3L mutations. One of the hyperactive mutations makes K3L more similar to eIF2α at a residue adjacent to the phosphorylation site in eIF2α at position 51. Loss-of-function K3L mutations reveal that a sequence in the C-terminal portion of K3L homologous to residues 79-83 in eIF2α (KGYID) , is critically required for K3L function. Biochemical analysis indicates that these K3L mutations exert their effects on PKR function by altering the affinity of K3L for PKR. These results support the notion that residues conserved between the pseudosubstrate K3L and the authentic substrate eIF2α play an important role in substrate recognition by PKR.

A novel regulator of eIF2α kinases. In searching the databases for other potential regulators of PKR, Dever and co-workers discovered a protein encoded by baculovirus called pk2 that is most similar in sequence to a truncated eIF2α kinase domain. They showed that expression of pk2 in yeast inhibits phosphorylation of eIF2α by both GCN2 and PKR, suggesting that insect cells contain an eIF2α kinase that is antagonized by pk2 during virus infection. Interestingly, the corresponding C-terminal portion of the GCN2 kinase domain can also function as a dominant inhibitor of wild-type GCN2 and PKR in yeast. They found that overexpression of a catalytically inactive form of PKR neutralized the ability of pK2 to inhibit GCN2, suggesting that the mutant PKR can bind to and sequester pk2. These results support the idea that pk2 inhibits PKR by complex formation. To gain evidence that pk2 functions in the virus as a means of down-regulating eIF2α phosphorylation during infection, they showed that expression of PKR in insect cells inhibits infection by the pk2-deleted virus relative to wild-type virus, and that infectivity of the pk2-deficient virus was rescued by expressing pk2 in *trans*.

Tyrosine phosphorylation by PKR in vivo. To date, the only proven substrate of PKR is eIF2; however, there are indications that other proteins may be phosphorylated by PKR in human cells, and the mouse homologue of PKR (TIK) was first identified in a screen for tyrosine kinases. Dever and his colleagues have shown that PKR can phosphorylate eIF2α on a Tyr residue at position 51, and will also autophosphorylate on Tyr residues when activated in yeast or human cells. These results show that PKR is a dual-specificity kinase and raise the interesting possibility that additional substrates for PKR exist in human cells which are phosphorylated on tyrosine rather than serine or threonine. Such substrates could be involved in growth regulation or signal transduction.

Function of an IF2-homologue in yeast translation initiation. Yeast encodes a protein called FUN12 related to the prokaryotic translation initiation factor IF2. In bacterial cells, IF2 stimulates binding of initiator $tRNA^{Met}$ to ribosomes, the same function carried out by eIF2 in conventional translation initiation in eukaryotic cells. Dever and his colleagues are investigating the possibility that FUN12 replaces eIF2 in an alternative initiation mechanism in eukaryotic cells, such as internal initiation or reinitiation. Results thus far suggest a requirement for FUN12 in efficient reinitiation on *GCN4* mRNA, in cap-independent translation initiation on the L-A or M viral mRNAs, and internal initiation directed by the leader of the *TIF4631* mRNA encoding eIF4G. It now appears that mammalian cells contain a FUN12 homologue, raising the possibility that this protein has an evolutionarily conserved function in cap-independent translation initiation.

TRANSPOSITION OF RETROELEMENTS IN FISSION YEAST

Retroelements including retroviruses and retrotransposons are widely distributed in nature. The great importance of these elements in biology at large makes it imperative to take advantage of the possibilities offered by yeast molecular genetics in their study. The research program conducted by ***Henry Levin*** focuses on the mechanisms of retroelement replication and integration into the host genome. Their approach to understanding the complex interactions between the retroelement and its host has been to study retrotransposons, a family of elements closely related to retroviruses. A significant advantage of studying retrotransposons is that they exist in hosts such as the yeast *Schizosaccharomyces pombe* which can be manipulated using sophisticated genetic techniques.

Self-priming mechanism of the Tf1 Retroelement of Fission Yeast. Levin and colleagues previously obtained strong genetic evidence that the transposon Tf1 uses a novel self-priming mechanism to initiate cDNA synthesis wherein the first 11 nucleotides of the Tf1 transcript anneal to the primer binding site. They obtained biochemical evidence supporting this mechanism by which is determining the 5' and 3' ends of the "minus-strand strong-stop DNA", which is the initial product of reverse transcription beginning at the primer binding site and extending to the beginning of the RNA transcript. By making use of Tf1 mutations which they generated in the active site of the reverse transcriptase to trap priming intermediates, Levin and colleagues obtained evidence for the existence of Tf1 transcripts missing the first 11 nucleotides. These results did not eliminate the possibility of a 2'OH linkage between RNA and the DNA strand of the strong-stop extension product at the 11th nucleotide of the Tf1 transcript. Accordingly, they have now used a "splint ligation" assay to prove definitively that the Tf1 primer is cleaved between the 11th and 12th bases. These data support their model that Tf1 priming involves cleavage of the first 11 nucleotides of the Tf1 transcript followed by reverse transcription from the 3' OH of the cleaved transcript. Unexpectedly, they found that mutations D710N and D779N in the RNAse H domain of Tf1 impaired cleavage at the 11th base of the Tf1 transcript, suggesting a novel role for RNAse H in cleaving an RNA-RNA duplex.

Analysis of a large set of point mutations near the primer binding site (PBS) has revealed the existence of a 39 nucleotide region surrounding the PBS that is essential for transposition. In addition to the 11-base pair duplex formed at the PBS, flanking 5-base pair and 11-base pair helices also appear to be critical for priming of reverse transcription. The importance of these additional helical regions was established by showing that compensatory mutations that restore the duplex structures also rescued the production of reverse transcripts. The mutations in the flanking helices impair strong-stop DNA synthesis and cleavage of the Tf1 transcript at the 11th base, the latter indicating an inability to generate the primer for reverse transcription. Thus, a complex RNA structure appears to be required to generate the substrate for RNAseH cleavage of the Tf1 transcript which produces the priming 3'OH for reverse transcriptase. Interestingly, a family of 8 retrotransposons in various fungi has been identified that appear to use a self-priming mechanism similar to that elucidated for Tf1.

RNAse H mutations that impair transposition but not reverse transcription. Random mutagenesis of Tf1 has led to the identification of several mutations in the integrase domain that reduce transposition without decreasing the level of reverse transcripts, as expected for a lesion in the integration reaction. Surprisingly, the same phenotype has been observed for mutations in the RNAseH domain, raising the possibility that this region also plays a role in integration.

Genetic analysis of host genes involved in Tf1 transposition. To identify host factors required for Tf1 transposition, a large number of mutant strains were isolated that are defective for transposition. At present, they are focusing on three mutants. The *hop2* mutant shows low levels of Tf1 proteins but normal levels of Tf1 mRNA, indicating a post-transcriptional defect in gene expression. The *hop3* and *hop5* mutants exhibit normal Tf1 protein expression and reverse transcript production, suggesting an integration defect. Efforts are underway to determine whether the *hop3* and *hop5* mutants have general defects in mitotic recombination and to clone the *HOP3* gene.

BIBLIOGRAPHY

Atwood A, Lin J, Levin HL. The retrotransposon Tf1 assembles virus-like particles with excess Gag due to specific degradation of Pol protein, Mol Cell Biol 1996;16:338-46.

Dever TE. Analysis of eIF2α kinases in yeast. In: Martin R, ed. Methods in Molecular Biology: Protein Synthesis: Methods and Protocols. Totawa, NH: Humana Press Inc., in press.

Dever TE. Using *GCN4* as a reporter of eIF2α phosphorylation and translational regulation in yeast, Methods, in press.

Dever TE, Yang W, Astrom S, Bystrom AS, Hinnebusch AG. Modulation of $tRNA_i^{Met}$, eIF2 and eIF2B expression shows that *GCN4* translation is inversely coupled to the level of the eIF2•GTP•Met-$tRNA_i^{Met}$ ternary complexes, Mol Cell Biol 1995;15:6351-63.

Grant CM, Miller PF, Hinnebusch AG. Sequences 5' of the first upstream open reading frame in *GCN4* mRNA are required for efficient translational reinitiation, Nuc Acids Res, 1995;23:3980-8.

Hinnebusch AG. Translational control of *GCN4:* gene-specific regulation by phosphorylation of eIF2. In: Hershey JWB, Mathews MB, Sonenberg N, eds. Translation control. New York: Cold Spring Harbor Laboratory Press, 1996;199-244.

Jackson BM, Drysdale CM, Natarajan K, Hinnebusch AG. Identification of seven hydrophobic clusters in GCN4 making redundant contributions to transcriptional activation. Mol Cell Biol, in press.

Levin HL. An unusual mechanism of self-primed reverse transcription requires RNAse H for cleavage of an RNA duplex, Mol Cell Biol, in press.

Taylor DR, Lee SB, Romano PR, Marshak DR, Hinnebusch AG, Esteban M, Matthews MB. Autophosphorylation sites participate in the activation of the double-stranded RNA-activated protein kinase PKR. Mol Cell Biol, in press.

Yang W, Hinnebusch AG. Identification of a regulatory subcomplex in the guanine nucleotide exchange factor eIF2B that mediates inhibition by phosphorylated eIF2. Mol Cell Biol, in press.

Project Numbers - Annual Report 1996

LABORATORY OF EUCARYOTIC GENE REGULATION (LEGR)

Z01 HD 01004-13	Regulation of Amino Acid and Nucleotide Biosynthesis in Saccharomyces Cerevisiae Alan G. Hinnebusch, Ph.D.
Z01 HD 01009-04	Regulation and Function of Retroelements Henry Levin, Ph.D.
Z01 HD 01010-02	Regulation of Eukaryotic Protein Synthesis Thomas E. Dever, Ph.D.

Z01 HD 00924-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01004-13 LEGR

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Amino Acid and Nucleotide Biosynthesis in Saccharomyces cerevisiae

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: A.G. Hinnebusch, Research Microbiologist, (All listed personnel LEGR:NICHD)

M. Garcia	Vis. Fellow	M. Marton	IRTA Fellow	G. Pavitt	Vis. Fellow
C. Vasquez	Vis. Fellow	P. Romano	IRTA Fellow	J. Anderson	IRTA Fellow
W. Yang	Vis. Assoc.	R. Rolfes	IRTA Fellow	L. Phan	NRC Fellow
B. Jackson	Biologist	K. Natarajan	Vis. Fellow	H. Qiu	IRTA Fellow
C. Drysdale	IRTA Fellow	F. Zhang	Guest Researcher	K. Asano	Vis. Fellow

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Eukaryotic Gene Regulation

SECTION

Section on Nutrient Control of Gene Expression

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
13.50	13.50	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

GCN4 is a transcriptional activator of amino acid biosynthetic genes that is regulated at the translational level by the eIF-2α kinase GCN2. Phosphorylation of eIF-2 by GCN2 under amino acid starvation conditions stimulates *GCN4* translation by inhibiting guanine nucleotide exchange on eIF-2 by eIF-2B, reducing the concentration of the ternary complex eIF-2/GTP/Met-tRNA$_i^{Met}$. Genetic and biochemical analysis has shown that the GCN3, GCD7, and GCD2 subunits of eIF-2B comprise a regulatory subdomain that mediates inhibition of eIF2B by phosphoryated eIF2. These proteins contain two 70-residue homologous segments that are critcally required for their regulatory functions. GCD10 is a component of the eIF-3 complex and *gcd10* mutations are suppressed by extra copies of genes encoding Met-tRNA$_i^{Met}$ (*IMT*) or the yeast homologue of La autoantigen (*LHP1*). *gcd10* mutants accumulate low levels of Met-tRNA$_i^{Met}$, but normal levels of all other tRNAs, and extra copies of *IMT* or *LHP1* restore Met-tRNA$_i^{Met}$ to nearly wild-type levels in *gcd10* mutants. GCD10 has been localized to the nucleus by immunofluorescence, and we propose that GCD10 interacts with Met-tRNA$_i^{Met}$ in the nucleus and delivers it to eIF3 in the cytoplasm where it can be utilized in ternary complex formation. GCN1 and GCN20 comprise a protein complex required to activate GCN2 kinase function in starved cells. An N-terminal segment of GCN20 binds to a domain in GCN1 related to translation elongation factor 3 (EF-3). GCN20 contains ATP-binding cassettes found in membrane transporters; however, immunofluorescence shows that GCN1 is distributed throughout the cytoplasm, and both GCN1 and GCN20 bind to polyribosomes in an ATP-stimulated fashion. We propose that GCN1/GCN20 function on the ribosome to facilitate delivery of uncharged tRNA to GCN2. Thr residues located between kinase subdomains VII and VIII in GCN2 are required for kinase activity in vivo and are sites of autophosphorylation in vitro. Conserved Thr residues in the human eIF2α kinase PKR are also critcally required for PKR function in vivo. Fragments of GCN2 containing the protein kinase o C-terminal ribosome-binding domains can form homo- or heterodimers in vivo, and we suggest that binding of the C-terminal domain to the kinase moiety regulates GCN2 function. Binding of uncharged tRNA to a histidyl-tRNA synthetase-related domain in GCN2 would alter the interaction between the C-terminal and kinase domains in a way that stimulates GCN2 activity. The transcriptional activation domain of GCN4 contains 7 clusters of bulky hydrophobic amino acids essential for transcription in vivo. These sequences are also required for in vitro binding of GCN4 to TBP-associated factors TAF$_{II}$60 and TAF$_{II}$90, suggesting that GCN4 activates transcription by recruiting the TBP-containing complex TFIID to the promoter. Transcriptional repression of the *ADE5,7* gene by adenine involves down-regulating complex formation by the BAS1 and BAS2 activator proteins at the promoter. Interaction with BAS2 stimulates a latent activation domain in BAS1. A *BAS1* mutation has been obtained that appears to permit BAS1/BAS2 complex formation in adenine-replete cells.

Project Description

Objectives:

To understand at the molecular level how the yeast *S. cerevisiae* regulates the expression of genes encoding enzymes involved in synthesizing amino acids and nucleotides according to the availability of these nutrients in the environment. One facet of this regulation, known as general amino acid control, involves increased expression of a large number of amino acid biosynthetic genes in response to starvation for any amino acid. Synthesis of GCN4 protein, the transcriptional activator in this system, is stimulated under starvation conditions by a translational control mechanism involving short open reading frames (uORFs) in the *GCN4* mRNA leader, several general translation initiation factors, and a protein kinase known as GCN2. A combination of genetic, molecular and biochemical approaches are being used to identify and characterize the trans-acting factors, both positive and negative, that mediate the regulatory functions of the uORFs. Some of these factors are subunits of the general translation initiation factors eIF-2 or its guanine nucleotide exchange factor eIF-2B; others appear to be components of eIF3 or previously unidentified initiation factors; still others, including the protein kinase GCN2 and its positive effectors GCN1 and GCN20, are regulatory factors dedicated to *GCN4* control. We wish to identify the biochemical functions of each factor and determine its position in the signal-transduction pathway that detects uncharged tRNA in amino acid-starved cells and modifies the translational machinery in a way that stimulates *GCN4* expression. This regulatory mechanism involves phosphorylation of the α subunit of eIF-2 by the protein kinase GCN2 and consequent reduction in the ability of eIF-2B to recycle eIF-2. This same mechanism operates in mammalian cells to inhibit total protein synthesis in response to various stress conditions, including amino acid starvation. By combining the powerful genetics and molecular biology of yeast with the biochemical analysis of translation initiation, we hope to provide a detailed molecular description of this highly conserved mechanism for regulating protein synthesis. The human eIF-2α kinase PKR (double-stranded RNA-activated inhibitor of translation) is an important regulator of cell growth and differentiation and a critical component of the interferon response to viral infection in humans. We have shown that PKR can functionally substitute for GCN2 in yeast cells and we are using the *GCN4* genetic system to determine how PKR is activated by dsRNA and to elucidate the mechanism whereby vaccinia virus E3L protein prevents activation.

In addition to studies on translational control, we are attempting to elucidate the molecular mechanism of transcriptional activation by GCN4. This involves an in-depth mutational and structural analysis of the transcriptional activation domain of GCN4, and probing with biochemical and genetic techniques for sequence-specific interactions between the GCN4 activation domain and general transcription factors. We are also studying transcriptional control of adenine biosynthetic genes and have identified the minimal cis-acting sequences at the *ADE5* gene necessary for adenine repression as binding sites for BAS1, BAS2 and ABF1. BAS1 and BAS2 form a complex at the promoter, and the formation and function of this complex appear to be down-regulated by adenine. We are attempting to define in molecular terms the interactions these proteins make with one another, with the transcriptional machinery, and with the upstream regulators that receive and transduce signals of adenine availability. Parallel studies of general amino acid control and the regulation of nucleotide biosynthesis in yeast should provide an integrated view of how eukaryotic cells control the levels of substrates (amino acids and nucleotides) and the machinery for protein and nucleic acid synthesis in the face of a changing nutritional environment.

Major Findings

I. Function of translation initiation factors that control *GCN4* expression.

A. Identification of a regulatory domain in eIF2B that mediates its inhibition by phosphorylated substrate.

Phosphorylation of the α subunit of translation initiation factor-2 (eIF-2α) on residue Serine-51 is a prominent mechanism for regulating protein synthesis eukaryotic cells. Inhibition of translation initiation by phosphorylation of eIF2 occurs by down-regulation of eIF2B, the 5-subunit guanine nucleotide exchange factor for eIF2. Deletion of the only nonessential subunit of eIF2B (encoded by *GCN3)* decreases the toxicity of eIF2(αP), implicating GCN3 in negative regulation of eIF2B. Previously, we described several amino acid changes in the *GCD7* (ß) subunit and one point mutation in the *GCD2* (δ) subunit of eIF2B that phenotypically mimic deletion of *GCN3*. We have now characterized many additional mutations in *GCD2* (9 alleles), *GCD7* (11 alleles) and *GCN3* (9 alleles) that decrease or abolish inhibition of eIF2B by eIF2(αP) in a dominant fashion. Genetic and biochemical data indicate that most, if not all, of the *GCD7* and *GCD2* mutations decrease sensitivity to eIF2(αP) without eliminating GCN3 from the eIF2B complex. In addition, the mutations have no effect on cell growth when eIF2 is unphosphorylated, suggesting that the catalytic function of eIF2B is unaffected. These results indicate that each of the 3 subunits plays a critical role in the negative regulation of eIF2B by eIF2(αP). Interestingly, the majority of the regulatory mutations in *GCD2, GCD7* and *GCN3* are clustered in 2 regions of sequence similarity ca. 70-amino acids in length at the amino and carboxyl ends of the homologous domains in all 3 proteins. These results strongly suggest that structurally related segments in GCD2, GCD7 and GCN3 carry out similar roles in the regulation of eIF2B by eIF2(αP). Several mutations mapping in the C-terminal cluster of regulatory mutations altered amino acids at the same positions in 2 or all 3 subunits. For the N-terminal clusters of mutations, the substitutions occurred at unique positions in each subunit. We propose that GCD2, GCD7 and GCN3 use homologous segments to make distinct contacts with eIF2α in the vicinity of the phosphorylation site, and that these interactions are critical for the inhibition of eIF2B function by eIF2(αP). The fact that most of the eIF2 is phosphorylated in certain *GCD2* regulatory mutants, suggests that these mutations allow eIF2B to accept phosphorylated eIF2 as a substrate.

The isolation of regulatory mutations affecting homologous residues in GCD2, GCD7, and GCN3, led us to propose that these proteins comprise a regulatory domain that interacts with eIF2(αP) and mediates the inhibition of eIF2B activity. Supporting this hypothesis, we found that overexpression of GCD2, GCD7 and GCN3 specifically reduced the inhibitory effect of eIF2(αP) on translation initiation in vivo. The excess GCD2, GCD7 and GCN3 were co-immunoprecipitated from cell extracts, providing physical evidence that these 3 proteins can form a stable subcomplex. Formation of this subcomplex did not compensate for a loss of eIF2B function by mutation, and in fact, lowered eIF2B activity in strains lacking eIF2(αP). These findings indicate that the trimeric subcomplex does not possess guanine nucleotide exchange activity and we propose, instead, that it interacts with eIF2(αP) and prevents the latter from inhibiting native eIF2B. Overexpressing only GCD2 and GCD7 also reduced eIF2(αP) toxicity, presumably by titrating GCN3 from eIF2B and producing the 4-subunit form of eIF2B that is less sensitive to eIF2(αP). This interpretation is supported by the fact that overexpressing GCD2 and GCD7 did not reduce eIF2(αP) toxicity in a strain lacking *GCN3;* however, it did suppress the impairment of eIF2B caused by the *gcn3^c-R104K* mutation. An N-terminally truncated GCD2 protein interacted with other eIF2B subunits only when GCD7 and GCN3 were overexpressed, in accordance with the idea that the portion of GCD2 homologous to GCD7 and GCN3 is sufficient for complex formation by these proteins. Together, our results provide strong evidence that GCN3, GCD7 and the C-terminal half of GCD2 comprise the regulatory domain in eIF2B.

B. GCD10, the RNA binding subunit of eIF-3, is required for accumulation of the ternary complex eIF2/GTP/Met-tRNA$_i^{Met}$ in vivo.

Mammalian eIF-3 is an 8-subunit complex that stimulates several steps in the initiation pathway in cell-free translation systems. Biochemical studies on mammalian eIF-3 have implicated this factor in several steps of initiation, including dissociation of 80S ribosomes into free 60S subunits and 46S preinitiation complexes containing eIF-3 and 40S subunits, stabilization and binding of eIF-2/GTP/Met-tRNA$^{Met}_i$ ternary complexes to 46S complexes, and binding of mRNA to 43S complexes containing eIF-3 and the ternary complex. Yeast contains a structurally similar complex that can functionally replace mammalian eIF-3 in an in vitro translation system containing all the initiation factors from Hela cells except for eIF-3. Previously, we obtained strong biochemical evidence that *GCD10* encodes the 54.6kDa RNA-binding subunit of yeast eIF-3. Mutations in *GCD10* lead to constitutive derepression of *GCN4* translation, the same phenotypes associated with *gcd* mutations affecting subunits of eIF-2 or eIF-2B. Translation of *GCN4* is repressed in nutrient-rich medium by 4 short uORFs present in the mRNA leader. Ribosomes bypass the uORFs and translate *GCN4* in amino acid-starved cells because of a reduction in levels of ternary complexes brought about by phosphorylation of eIF2. Based on their effect on *GCN4* translation, we proposed that *gcd10* mutations reduce the ability of eIF-3 to rebind to 40S subunits, or decrease the ability of eIF-3 to stimulate binding of eIF-2/GTP/Met-tRNA$^{Met}_i$ ternary complexes to 46S complexes rather than delaying the formation of these complexes. Either of these last two defects would decrease the rate at which ternary complexes can rebind to 40S subunits scanning and explain why *gcd10* mutations cause ribosomes to ignore uORFs 2-4 and reinitiate at *GCN4* in the absence of eIF-2 phosphorylation by GCN2.

gcd10 mutations also confer temperature-sensitive growth on rich medium resulting from the defect in general translation initiation. We found that *gcd10* mutants at the non-permissive temperature contain tRNA$_i^{Met}$ at levels ca. 6-fold lower than in wild-type, whereas elongator tRNA^{Met} and several other tRNAs were unaffected. No reductions in tRNA$_i^{Met}$ levels were seen in an eIF2α mutant; therefore, it is not a general consequence of impaired initiation. The levels of precursor tRNA$_i^{Met}$ transcripts were not reduced in *gcd10* mutants, implying that the defect in accumulation of mature tRNA$_i^{Met}$ occurs post-transcriptionally. Unexpectedly, the majority of GCD10 was localized to the nucleus of cells using indirect immunofluorescence. The temperature-sensitive growth and derepression of *GCN4* translation associated with *gcd10* point mutations was suppressed by increasing the dosage of any of the 4 genes encoding tRNA$_i^{Met}$ (*IMT1-4*), leading to ca. 5-fold higher levels of tRNA$_i^{Met}$ compared to the parental strains. Remarkably, high copy-number *IMT* genes also suppressed the lethality of a *gcd10* deletion. These results provide strong evidence that promoting tRNA$_i^{Met}$ accumulation is the essential function of *GCD10*. This conclusion is supported by the isolation of a second dosage suppressor, *LHP1*, encoding the yeast homologue of the human La autoantigen. Overexpression of *LHP1* in *gcd10* mutants also led to increased levels of tRNA$_i^{Met}$, in accordance with published data indicating that La functions in the expression or processing of RNA polymerase III transcripts. Interestingly, expression of La from humans or *Schizosaccharomyces pombe* suppressed the lethality of *gcd10* mutations, providing *in vivo* evidence that LHP1 is a functional homologue of the La autoantigen. We propose a model in which GCD10 functions in the nucleus to stabilize tRNA$_i^{Met}$ during nascent transcript processing or transport to the cytoplasm. GCD10 may then interact with other subunits of eIF3 as a complex with tRNA$_i^{Met}$, delivering the initiator to the site of ternary complex formation in the cytoplasm.

C. Physical association between GCD14 and GCD10.

We previously isolated mutations in the *GCD13, GCD14* and *GCD15* genes that have the same phenotypes as the mutations affecting subunits of eIF2, eIF-2B or GCD10. The *GCD14*gene was cloned and found to encode a protein of ca. 40 kDa that contains a degenerate RNA recognition motif (RRM), and thus could be an RNA binding protein. Based on peptide sequencing, GCD14 is not a subunit of the eIF3 complex purified in John Hershey's laboratory. However, we found that GCD14 co-purifies with a His-tagged form of GCD10 on Ni-affinity columns and co-immunoprecipitates with an HA epitope-tagged form of GCD10 from yeast extracts. Using a His-tagged form of PRT1, a 90 kDa subunit of yeast eIF3, we confirmed that GCD10 is a component of yeast eIF3; however, the association of GCD14 with His-PRT1 appears to be salt-labile. These results indicate that GCD14 is physically associated with the *GCD10* subunit of eIF3, and based on the phenotype of *gcd14* mutations, may also be required for ternary complex accumulation. GCD14 appears to be more loosely associated with eIF3 than are other subunits of eIF3. Using the His-tagged PRT1 protein, we also showed that the yeast *NIP1* protein co-purifies with the eIF3 complex. NIP1 is homologous to a subunit of human eIF3; however, until now, it appeared that NIP1 was not a subunit of yeast eIF3.

II. Regulation of the protein kinase GCN2 by nutrient availability.

A. Role of autophosphorylation and domain interactions in the activation of GCN2 kinase function in amino acid starved cells.

The protein kinase GCN2 stimulates translation of *GCN4* mRNA and down-regulates general protein synthesis in yeast cells starved for amino acids or purines by phosphorylating the α subunit of eIF2 on Ser-51. Several functional domains have been defined in GCN2, including the kinase catalytic domain, a pseudo-kinase domain, a histidyl-tRNA synthetase-related (HisRS) region, and the extreme C-terminal segment that is required for ribosome-association of GCN2. Mutations that constitutively activate GCN2 (*GCN2*^{c} alleles) have been obtained in the catalytic domain, HisRS-related region and extreme C-terminus, strongly implicating these regions in regulation of GCN2 function. The HisRS-related region binds tRNA in vitro, supporting the idea that uncharged tRNA which accumulates in amino acid-starved cells binds to this domain as the means of stimulating GCN2 activity. The yeast two-hybrid system was employed to examine the possibility that the different domains in GCN2 physically interact with one another. We found that a segment containing ca. 2/3 of the protein kinase domain, and another containing the C-terminal region, interacted with themselves in the two-hybrid assay. These results suggest that GCN2 dimerizes through interactions involving the protein kinase or C-terminal domains. Initial experiments indicate that a LexA fusion protein containing only the C-terminal segment of GCN2 co-immunoprecipitates with wild-type GCN2, providing biochemical support for this conclusion. Additional genetic evidence for dimerization comes from the observation that a catalytically defective GCN2 protein has a dominant-negative effect on the function of GCN2^c proteins.

By constructing serial deletions in the C-terminal segment of GCN2, we found that 2 non-contiguous segments of ca. 60 and 20 amino acids were important and essential, respectively, for dimerization in the two-hybrid assay. Deleting each of these 2 segments in full-length *GCN2* abolished induction of *GCN4* translation in vivo, supporting the idea that dimerization is important for GCN2 activation or catalytic function. Mutating 3 lysine residues required for ribosome-binding by GCN2 and for the in vitro RNA-binding activity of the C-terminal segment (S. Zhu and R. Wek, unpublished observations) did not affect its ability to dimerize. The protein kinase and C-terminal segments also interacted with one another in the two-hybrid assay, suggesting that the C-terminus directly interacts with the catalytic domain in regulating GCN2 kinase function. We speculate that mutually exclusive interactions between the C-terminal segments with one another or with the protein kinase domains in a GCN2 homodimer are involved in the regulation of kinase activity by uncharged tRNA.

Many protein kinases are activated by phosphorylation of one or more residues in a loop located between kinase subdomains VII and VIII, involving either autophosphorylation or phosphorylation by another kinase. We have found that GCN2 autophosphorylates in vitro on 2 Thr residues in this "autophosphorylation loop" and that the presence of phosphorylatable residues at these positions are critical for GCN2 function in vivo. These results suggest that one aspect of GCN2 activation involves autophosphorylation. GCN2 is also phosphorylated at other sites in vivo, suggesting that its kinase activity is modulated by other protein kinases.

B. GCN1 and GCN20 are components of a heteromeric protein complex that functions on the ribosome to activate GCN2 in amino-acid starved cells.

Previously, we showed that GCN2 is a ribosome-associated protein kinase that phosphorylates eIF2 in amino acid starved cells and thereby stimulates translation of *GCN4* mRNA. Uncharged tRNA is believed to activate GCN2 by binding to a regulatory domain in the kinase related to histidyl-tRNA synthetase. GCN1 and GCN20 reside in a protein complex required for activation of GCN2 and consequent phosphorylation of eIF2 in amino acid-starved cells. GCN20 is a member of the ATP-binding-cassette (ABC) family of proteins, most of which function in membrane transport; however, the GCN20 ABC domains are highly related to those present in yeast translation elongation factor 3 (EF3). Interestingly, GCN1 also resembles EF3 in a region N-terminal to the ABC domains. Deleting 85% of GCN20 from the C-terminus (including the ABC domains) only partially impaired its regulatory function, at odds with the idea that GCN20 functions in membrane transport. The N-terminal portion of GCN20 is necessary and sufficient for interaction with GCN1 and the EF3-related domain of GCN1 is necessary and sufficient for interaction with GCN20 In addition, we isolated a point mutation in a conserved residue in the EF3-like domain of GCN1 that reduces complex formation with GCN20. These results underscore the importance of the sequence similarity between GCN1and EF3. Immunofluorescence reveals that GCN1 is distributed uniformly throughout the cytoplasm, and biochemical fractionation experiments indicate that the GCN1/GCN20 complex binds to polyribosomes. The ribosome-association of GCN20 is largely dependent on GCN1. Overexpression of GCN1 is toxic and confers increased sensitivity to the translational inhibitor paromomycin; it also exacerbates the toxicity of overexpressing elongation factor EF1α. Based on these results, we propose that the GCN1/GCN20 complex acts at the ribosome to detect uncharged tRNA in the A-site and to deliver this molecular signal to GCN2 for stimulation of kinase activity. Although ATP hydrolysis by GCN20 is dispensable for this regulatory function, it may serve to modulate the extent or kinetics of GCN2 activation, or to integrate kinase activation with the availability of other essential nutrients in the cell. We have identified human proteins homologous to GCN1 and GCN20 by database searches, and a human GCN2 homologue has been identified in another lab. These last findings suggest that the mechanism for activating an eIF2α kinase by uncharged tRNA is conserved between yeast and humans.

III. Regulation of the mammalian eIF-2α kinase PKR in yeast.

A. Analysis of the importance of autophosphorylation sites for kinase function in vivo.

In mammalian cells, stimulation of the latent protein kinase activity of the double-stranded-RNA-activated inhibitor of translation, PKR, occurs in response to virus infection. The N-terminus of PKR contains two copies of a sequence motif found in several dsRNA-binding proteins and numerous deletion and point mutations have been introduced into these sequences that affect dsRNA binding in vitro. We showed previously that PKR expressed at low levels in yeast mimics the function of GCN2 in stimulating translation of *GCN4*, and inhibits total protein synthesis when expressed at high levels. Exploiting these findings, we went on to demonstrate that the dsRNA-binding domain (DRBD) is required for PKR kinase function in yeast cells and that the dsRNA-binding motifs are involved in the activation mechanism.

We have been attempting to identify sites of autophosphorylation in PKR that are important for its activation by dsRNA in yeast cells. Mike Mathew's lab have identified several tryptic peptides from the N-terminal regulatory domain of PKR that are autophosphorylated in vitro by PKR purified from cultured human cells. In collaboration with their group, we have shown that alanine substitutions in the various Ser and Thr residues in these peptides reduce, but do not abolish PKR function in yeast. We have also shown that two Thr residues located in the autophosphorylation loop between subdomains VII and VIII are critical for PKR function both in vitro and in vivo. We are currently overproducing and purifying PKR from yeast cells to permit identification of the phosphopeptides produced by autophosphorylation in vivo using mass spectroscopy.

B. Mechanism of down-regulation of PKR by vaccinia E3L protein.

The E3L protein of vaccinia virus, which down-regulates PKR in infected mammalian cells, contains one DRBD in its C-terminal half that is required for dsRNA-binding in vitro and for complementing the interferon sensitivity of a vaccinia E3L deletion mutant. Biochemical experiments indicate that E3L can inhibit PKR activation in vitro by sequestering dsRNA. We found that expression of E3L reversed the growth-inhibition by PKR in yeast cells, and this activity was impaired by a mutation in the E3L DRBD that abolishes dsRNA-binding in vitro. Deletions in the N-terminal half of E3L also reduced or abolished its ability to inhibit PKR in yeast. One such mutant had a dominant-negative phenotype, apparently interfering with the ability of wild-type E3L to inhibit PKR. At present, it is possible that the effects of the N-terminal deletions derive, at least partly, from reductions in E3L protein expression. If not, then combining our results with previous observations indicating that the N-terminal half of E3L does not contribute to dsRNA-binding in vitro, would lead us to conclude that dsRNA-binding is not the sole function required by E3L to inhibit PKR in yeast. This would agree with our previous finding that overexpressing catalytically defective PKR-K296R did not reduce activation of wild-type PKR, suggesting that dsRNA is too abundant in yeast to be sequestered by the mutant PKR. The dominant interference of the E3L N-terminal truncation can be explained by proposing that E3L must physically interact with PKR to inhibit kinase activity in yeast and that the defective N-terminally truncated E3L protein competes with wild-type E3L for binding to PKR. These conclusions are supported by our finding that a substantial fraction of PKR can be specifically co-immunoprecipitated with epitope-tagged E3L from yeast cell extracts. Interestingly, neither the N-terminal truncation or the mutation in the E3L DRBD reduced coimunoprecipitation of PKR with E3L. These last results suggest that complex formation is independent of dsRNA-binding by E3L and is mediated by protein-protein interactions involving the E3L DRBD. Our model also explains the fact that inhibition of wild-type PKR by E3L was diminished by co-expressing the PKR-K296R protein, as the defective PKR would be expected to sequester E3L and neutralize its ability to complex with wild-type kinase. Together, our results suggest that inhibition of PKR by E3L in yeast requires complex formation between these two proteins, and identify a requirement for the E3L N-terminal domain in this regulatory mechanism.

IV. Mechanism of transcriptional activation by GCN4.

A. Mutational analysis of the GCN4 activation domain.

In previous work we showed that GCN4 contains at least two activation domains that are critically dependent on hydrophobic amino acids. Large segments of GCN4 could be removed without lowering transcriptional activation of *HIS3* and *HIS4*, and either of two domains, called the N-terminal (NTAD) and central acidic (CAAD) activation domains, could activate transcription with similar efficiencies. Activation by the CAAD (in the absence of the NTAD) was dependent on 2 clusters of hydrophobic residues: M-107, Y-110, L-113 and W-120, L-123, F-124 (lines 8-12), and activation by the NTAD (in the absence of the CAAD) was dependent on the aromatic cluster F-97, F-98 (lines 13-18). It was necessary to mutate all 3 clusters in the NTAD and CAAD to greatly impair activation of the *HIS4* promoter (lines 19-22).

We suspected that the NTAD contained additional residues critical for its function and set out to identify them by random mutagenesis of a *GCN4* allele lacking the entire CAAD. Our results indicate that the NTAD contains 3 additional pairs of Phe residues in addition to F-97/F-98, namely F-9/F-16, F-45/F-48 and F-67/F-69, plus L-89, all of which are critical for activation when the CAAD is missing. Substitutions in numerous positions flanking the critical residues had little or no effect on activation. Trp, Leu and Tyr were found to be highly functional substitutions for the Phe residue at position 45. Immunoblot analysis showed that the reductions in *GCN4* function caused by substitutions of critical residues were not the result of reduced GCN4 protein levels. Combined with our previous findings, these results indicate that GCN4 contains 7 clusters of aromatic or bulky hydrophobic residues which all make important contributions to transcriptional activation.

By combining point mutations in the critical hydrophobic clusters in different combinations we obtained evidence for extensive functional redundancy among these clusters. None of the 7 hydrophobic clusters is essential for activation by full-length GCN4, and the critical residues in 2-3 clusters had to be mutated simultaneously to observe a substantial reduction in *GCN4* function. The only double substitution that strongly impaired activation involved the clusters at 97/98 and 120/123/124, suggesting that these are particularly important constituents of two functionally redundant activation domains. It appears that the clusters at positions 45/48/67/69 and 97/98 belong to 2 distinct domains, rather than being constituents of a single domain (the NTAD). In fact, we can account for our results by proposing that all 7 hydrophobic clusters act independently to provide redundant means of activating transcription and that their functions are cumulative at the promoter. Based on these results, we propose that the hydrophobic clusters in GCN4 mediate multiple hydrophobic interactions with different components of the transcription initiation machinery.

B. GCN4 binds specifically to yTAF$_{II}$90 and yTAF$_{II}$60 in whole cell extracts.

To test the idea that the hydrophobic clusters in GCN4 are points of contact between the activation domain and specific transcription factors, we expressed GST-GCN4 fusion proteins containing wild-type or mutant activation domains in *E. coli* and tested them for the ability to interact with various transcription factors in yeast whole-cell extracts. Our results indicate that $yTAF_{II}90$ and $yTAF_{II}60$, two components of the TBP-containing transcription factor TFIID, bound to GST-GCN4 in a manner that depended on critical residues in the GCN4 activation domain. We found no significant binding of $yTAF_{II}130$ or TBP itself to the GST-GCN4 fusion proteins. The results of co-immunoprecipitation experiments indicated that substantial fractions of $yTAF_{II}90$, $yTAF_{II}60$, $yTAF_{II}130$, and a small fraction of TBP, were present in the same complexes in these extracts. To explain the fact that $yTAF_{II}90$ and $yTAF_{II}60$ bound to GST-GCN4 whereas $yTAF_{II}130$ and TBP did not, we propose that the $yTAF_{II}$ complex dissociated during the washing steps of the GST pull-down assays and that GCN4 bound tightly to a subcomplex containing $yTAF_{II}90$ and $yTAF_{II}60$ but lacking $yTAF_{II}130$ and TBP. We propose that the TFIID complex is an important target of the GCN4 activation domain and that GCN4 interacts directly with a subset of the components of this factor (including $yTAF_{II}90$ and $yTAF_{II}60$) as a means of stimulating assembly of the transcription initiation complex.

V. Genetic analysis of the regulation of purine biosynthetic genes.

A. Identification of the minimal cis-acting elements in the *ADE5,7* promoter required for adenine-repressible transcription.

The BAS1 and BAS2(PHO2) proteins are required for high-level expression of adenine biosynthetic genes in minimal medium, and the repression of *ADE* genes by excess adenine involves down-regulating the ability of BAS1 or BAS2 to activate transcription. BAS1 binds to DNA via a Myb-related domain and BAS2 is a homeodomain protein. We have identified a 74 bp fragment from *ADE5,7* containing two BAS1 binding sites separated by 33 bp that is sufficient to confer adenine-regulated transcription on the *CYC1* promoter. The proximal BAS1 site is absolutely required for UAS_{Ade} activity, whereas the distal BAS1 site only augments transcription dependent on the proximal site. By making clustered substitution mutations across the *ADE5,7* regulatory region, we found that an ABF1 site located between the two BAS1 sites is required for transcriptional activation in vivo, and mutations in these sequences abolished binding of ABF1 to the *ADE5,7* fragment in vitro. Substitutions 3' to the proximal BAS1 site also impaired transcriptional activation in vivo and weakened the binding of BAS2 protein to the *ADE5,7* sequences in vitro. Thus, the UAS_{Ade} at *ADE5,7* appears to consist of closely spaced binding sites for BAS1, BAS2 and ABF1. None of ca. 50 substitutions in the *ADE5,7* promoter produced the constitutively derepressed phenotype that would be indicative of a binding site for a putative repressor protein. Thus, it appears that adenine repression involves antagonism of the positive factors BAS1, BAS2 or ABF1 by protein modification or by interactions with a negative regulator that does not itself bind DNA. BAS1 is the most likely target for adenine regulation because ABF1 and BAS2 are required at other promoters (*PHO5* and *HO*) that are presumably unresponsive to adenine levels.

B. Recruitment of BAS2(PHO2) to the promoter by BAS1 is a key aspect of adenine-repressible transcription of *ADE5,7*.

We could envision several ways in which BAS1 function is regulated by adenine levels. Its localization in the nucleus could be prevented in adenine-excess, similar to the situation described for other factors, PHO4 and SWI5, that act in conjunction with BAS2. Alternatively, the ability of BAS1 to bind DNA, to recruit the activation domain of BAS2 to the promoter, or to stimulate transcription through its own activation domain could be down-regulated by adenine. It was also conceivable that an adenine-regulated repressor competes with BAS1 or BAS2 for their respective binding sites as the means of preventing activation under repressing conditions. To begin to distinguish between these possibilities, we fused BAS1 and BAS2 to the DNA binding domain of bacterial lexA and analyzed the fusion proteins for adenine-regulated transcriptional activation of a *lacZ* reporter containing lexA operator sites 5' of the TATA box. Expression of the lexA-BAS2 fusion protein in yeast cells conferred constitutive activation of a *lexA* reporter independently of *BAS1*. In contrast, a lexA-BAS1 fusion conferred adenine-repressible transcription of the *lexA* reporter in a manner highly dependent on *BAS2*. The ability of lexA-BAS1 to confer BAS2-dependent activation did not require the DNA binding activity of BAS2. These results suggest that complex formation by BAS1 and BAS2 is required to constitute a potent activator at the promoters of *ADE* genes; furthermore, they make it improbable that the DNA binding domains of BAS1 or BAS2 are targeted by the adenine repression mechanism. The activation functions of both authentic BAS1 and lexA-BAS1 were stimulated under adenine-repressing conditions by overexpression of BAS2. Inactivation of genomic *BAS1* had the same effect on the function of lexA-BAS1, as expected if lexA-BAS1 and authentic BAS1 compete for limiting amounts of BAS2 in adenine-replete cells. The fact that BAS2 is limiting for activation under repressing conditions is consistent with the idea that down-regulating complex formation by BAS1 and BAS2 is an important aspect of adenine repression. We isolated a dominant mutation in *BAS1* at residue Asp-617, located C-terminal to the DNA binding domain, that increases the ability of BAS1 or the lexA-BAS1 fusion to activate transcription in concert with BAS2 under repressing conditions. This mutation may act by reducing the negative effect of adenine on complex formation by BAS1 and BAS2. Deleting the C-terminal 22% of BAS1 from the lexA-BAS1 fusion protein led to high-level constitutive activation of the *lexA* promoter in a manner that was completely independent of *BAS2*. This last finding strongly suggests that BAS1 contains a latent activation domain that is functionally masked by the C-terminal segment under adenine repressing conditions, and that complex formation with BAS2 antagonizes the negative-acting domain and unmasks BAS1 activation function.

Significance to Biomedical Research and the Program of the Institute:

Yeast is a eukaryotic organism that carries out many of the fundamental processes of eukaryotic cells and offers a level of molecular genetic analysis that cannot be achieved with multicellular organisms. General amino acid control and the regulation of purine biosynthetic genes involve a variey of strategies for regulating gene expression. Investigating the molecular details of these two systems is providing useful paradigms for coordinate transcriptional activation of unlinked genes, transcriptional repression, gene-specific translational control, functional modifications of protein complexes involved in general translation initiation, and the involvement of protein kinases and protein phosphatases in signal-transduction pathways. The results of these inquiries can be expected to provide valuable insights into how higher eukaryotic cells control the availability of amino acid and nucleotide substrates for protein and nucleic acid synthesis in response to changes in the environment. In fact, *GCN4* translational control appears to be a gene-specific instance of a highly conserved mechanism that regulates protein synthesis in mammalian cells under conditions of stress such as amino acid starvation. The regulatory mechanisms under study in yeast are critical elements of the network of interrelated controls that couple growth rate and division to environmental stimuli in all eukaryotic cells.

Proposed Course of the Project.

B. Molecular genetic analysis of eIF-2B.

1. In vitro analysis of protein-protein interactions in the GCD2/GCD7/GCN3 subcomplex.

From immunoprecipitation experiments, we obtained biochemical evidence that GCD2, GCD7 and GCN3 can form a stable subcomplex in vivo. To test our prediction that the GCD2/GCD7/GCN3 subcomplex can interact with eIF-2, we are attempting to reconstitute the complex in vitro from proteins expressed in *E. coli* or translated in vitro, and probing for interactions with eIF-2 purified from yeast. With the higher protein concentrations that can be achieved with purified preparations, we hope to detect complexes that are not stable enough to be detected by immunoprecipitations from cell extracts. We have constructed plasmids for overexpression of GCD2, GCD7 and GCN3 in *E. coli* and we can now purify 1-2 mg of glutathione-S-transferase (GST)-tagged versions of GCD7, GCN3, and the C-terminal half of GCD2. In initial experiments, we found that GCD7 protein translated in vitro specifically interacts with the GST-GCD2 fusion protein in a manner that depends on residues in the C-terminal half of GCD7. In addition, this interaction was stimulated by addition of purified GCN3. If these results can be verified, they will provide biochemical evidence that GCD7, GCN3 and the C-terminal half of GCD2, which is homologous to the other two subunits can form a stable trimeric complex in the absence of any other yeast proteins. In addition, it will provide us with an assay that can be used to identify specific residues in each of the proteins required for their interactions with one another.

We will attempt to demonstrate binding of purified eIF-2 to this complex and analyze the effects of phosphorylating eIF-2 on the interaction. Wild-type eIF-2 is now being purified from a yeast strain containing a His-tagged form of *GCD11* and the genes encoding the other two subunits all contained on the same high copy plasmid. Using this strain, eIF2 can be highly purified from a ribosomal salt-wash using only Ni-affinity and heparin-agarose column chromotography. The eIF-2 will be applied to a glutathione column containing bound GST-GCD2/GCD7/GCN3 trimeric complex and its retention on the column will be analyzed by immunoblotting using antibodies against subunits of eIF-2. The K_D for the mammalian eIF-2/eIF-2B complex has been estimated between 0.1-1.0 nM (S. Kimball, personal communication). Thus, even if the K_D for interaction between eIF-2 and the GCD2-GCD7-GCN3 trimeric subcomplex is only 1 µM, we should be able to detect it using this technique if we bind ca. 25 µg of eIF2 to the trimeric subcomplex in a 50 µL reaction. We will establish the specificity of an interaction between eIF-2 and the eIF-2B subcomplex by showing that it depends on both GCD2 and GCD7 and that the affinity is higher when GCN3 is also present in the complex. It is possible that phosphorylation of eIF-2 will increase its affinity for the trimeric complex and that introduction of one or more Gcn$^-$ mutations into the GCD2, GCD7 or GCN3 proteins will diminish the affinity for phosphorylated eIF-2 or increase the affinity for nonphosphorylated eIF-2. An increase in affinity will be detected by retention of the same fraction of applied eIF-2 in the presence of less trimeric complex bound to the column.

2. Mapping protein-protein interactions among the subunits of yeast eIF2B with the yeast 2-hybrid approach.

Two libraries containing randomly sheared fragments of 300-1000 nt encoding different segments of the five subunits of eIF2B have been constructed in the GAL4-ACT and GAL4-DBD plasmids for two-hybrid analysis of subunit interactions. These libraries will be screened against one another to identify points of contact between the different subunits of eIF2B. Interactions detected by the two-hybrid assay will be verified in vitro by GST-pull down assays with the correponding protein fragments.

3. Biochemical analysis of regulatory mutations in subunits of eIF-2B.

The Gcn$^-$ regulatory mutations we isolated in *GCD2, GCD7* and *GCN3* could act to overcome the inhibition of eIF-2B function by eIF-2(αP) through any of the following mechanisms: (1) allowing eIF-2B to catalyze nucleotide exchange on eIF-2(αP); (2) decreasing the affinity of eIF-2B for eIF-2(αP); (3) increasing the affinity of eIF-2B for nonphosphorylated eIF-2; (4) increasing the rate of nucleotide exchange on the nonphosphorylated fraction of eIF-2. To distinguish between these possibilities, it will be necessary to measure dissociation constants, and the K_M and v_{max} for nucleotide exchange on eIF-2 using phosphorylated and nonphosphorylated eIF-2 and the mutant and wild-type versions of eIF-2B. Such measurements will require relatively large amounts of purified proteins, and to accomplish these goals, we have begun a collaboration with Scot Kimball's laboratory at Hershey Medical Center because of their expertise in purifying and characterizing eIF-2 and eIF-2B from rat liver. As described above, it is now relatively easy to purify large amounts of eIF2 using the yeast strain that overexpresses the eIF2 complex containing a His-tagged form of the γ subunit. Similarly, we have constructed a strain containing all five subunits of eIF-2B on two high-copy plasmids from which the eIF-2B complex is overproduced ca. 8-fold, and introduced His and HA tags at the C-terminus of the GCD1 (γ) subunit to permit affinity purification of the eIF-2B complex. We have now introduced Gcn$^-$ point mutations into the genes on the high copy plasmids encoding the α (*GCN3*) or β (*GCD7*) subunits of eIF-2B that should decrease the sensitivity of the eIF2B complex to the inhibitory effects of phosphorylating the substrate eIF2.

After purifying eIF2 and eIF2B from these engineered yeast strains, we will phosphorylate the eIF-2 in vitro using recombinant HRI and use it to determine the K_I for eIF-2(αP) for wild-type eIF2B by assaying rates of nucleotide exchange on eIF-2/[^{3}H]GDP binary complexes in the presence of varying concentrations of eIF-2(αP). We will then proceed to purify the mutant forms of eIF-2B bearing Gcn$^-$ mutations in the α or β subunits and measure K_M and v_{max} values using phosphorylated and nonphosphorylated eIF-2/[^{3}H]GDP binary complexes as substrate, and also K_I values, as described above. These experiments will reveal whether the mutant forms of eIF-2B differ from the wild-type complex in the ability to (1) catalyze nucleotide exchange more efficiently on phosphorylated eIF-2 or (2) catalyze exchange on nonphosphorylated eIF-2 in the presence of greater amounts of eIF-2(αP).

Kimball's lab also plans to measure dissociation constants for the eIF-2/eIF-2B and eIF-2(αP)/eIF-2B complexes using fluorescence spectroscopy after labeling the eIF-2 with the flourescent probe dansyl chloride (121). In this technique binding of purified eIF-2B to the labeled eIF-2 leads to a change in fluorescence anisotropy, which is measured at different concentrations of eIF-2B. In preliminary experiments, they used this technique to estimate the dissociation constant of eIF-2 and eIF-2B purified from rat liver at 1 nM. After measuring the K_D for the yeast eIF-2/eIF-2B complex, with and without prior phosphorylation of the eIF-2 by HRI, they will carry out the same measurements on a select group of purified mutant forms of eIF-2B containing various Gcn$^-$ regulatory mutations.

3. Studies on the regulation of mammalian eIF-2B by phosphorylated eIF-2.

There is a high degree of sequence similarity between GCD2, GCD7 and GCN3 and the corresponding subunits of mammalian eIF-2B. Interestingly, a number of the Gcn$^-$ alleles we isolated in these subunits alter residues that are conserved between the yeast and mammalian proteins. We wish to determine whether these amino acids are involved in regulating mammalian eIF-2B by eIF-2(αP) in the same way that they function in yeast. To test this idea, we are using site-directed mutagenesis to introduce the amino acids present in several Gcn$^-$ alleles of *GCN3* and *GCD7* into the corresponding positions of the rat eIF-2B cDNAs. These mutant cDNAs are being inserted into different vectors designed for overexpression in rat cells or in insect cells using baculovirus vectors. We will examine the effects of these mutations in subunits of rat eIF-2B on inhibition by phosphorylated eIF-2 in the following two ways.

In collaboration with Scot Kimball's lab the mutant and wild-type rat eIF-2B cDNAs will be expressed from the pBK-RSV vector in rat cells and analyzed for their effects on eIF-2B nucleotide exchange activity in cell extracts. They have established procedures for increasing eIF-2α phosphorylation by treating rat GH3 pituitary tumor cells with Brefeldin A and shown that eIF-2B in the whole cell extracts exhibits reduced nucleotide exchange activity for eIF-2/[^{3}H]GDP binary complexes added as substrate. We will determine whether overexpression of any of the mutant forms of eIF-2B subunits in the rat cells can rescue eIF-2B exchange activity. Kimball's group has also developed vectors for overexpressing all five subunits of rat eIF2B from baculovirus vectors in insect cells, and finds that the proteins assemble into complexes with eIF2B exchange activity. Thus, this system provides a second means of assaying the exchange activities of rat eIF2B complexes containing mutations predicted to reduce inhibition by phosphorylated eIF2.

C. Studies on the role of GCD10, GCD14, PRT1, and p39 in the structure and function of eIF-3.

1. Analysis of effects of *gcd10* and *gcd14* mutations on synthesis and stability of tRNA$_i^{Met}$.

We are carrying out pulse-chase experiments measuring incorporation of radioactive uracil into tRNA$_i^{Met}$ by hybridizing extracted total RNA to immobilized DNA encoding tRNA$_i^{Met}$. The *gcd10* mutants and wild-type *GCD10* strains grown at the permissive and nonpermissive temperatures are being compared in an effort to determine whether *gcd10* mutations affect the synthesis or stability of tRNA$_i^{Met}$. The same experiments will be carried out on *gcd14* mutants if we find that steady-state levels of tRNA$_i^{Met}$ are reduced in these strains as was found for the *gcd10* mutants.

2. Analysis of nuclear localization of GCD10 and GCD14.

We are analyzing HA-tagged forms of GCD14 and PRT1, the 90 kDa subunit of eIF3, to determine whether GCD14 is localized to the nucleus, and whether this characteristic is unique to the GCD10 subunit of eIF3. An HA-tagged form of the STE6 protein is being analyzed as a cytoplasmic control protein. We will also measure the relative amounts of GCD10, GCD14 and PRT1 in nuclear and cytoplasmic compartments by immunoblot analysis of extracts isolated from yeast nuclei or from intact cells. Wild-type and *gcd10* mutants will be compared in these experiments to determine whether the mutations affect nuclear localization of GCD10 and GCD14.

3. Domain structure of GCD10 and identification of its RNA binding site.

To begin our analysis of the role of RNA binding by GCD10 in eIF-3 function we will identify the minimal segment of GCD10 required for RNA binding in vitro and evaluate its importance for GCD10 function in vivo. We will construct nested deletions progressing from the N- and C-termini of GCD10 in a His-tagged allele tailored for expression in *E. coli* and for in vitro transcription/translation in rabbit reticulocytes. The mutant proteins will be tested for RNA binding by Northwestern analysis and by binding labelled RNA to the His-tagged GCD10 protein immobilized on Ni-columns. In these experiments we will also address the relative binding affinities of GCD10 for tRNA$_i^{Met}$, various other tRNAs, globin mRNA and 18S rRNA to determine whether the GCD10 RNA-binding domain shows any sequence specificity. In parallel experiments being done in collaboration with John Hershey's lab, we are mapping the RNA binding domain in the p66 subunit of human eIF3, and testing it for sequence specificity. This protein appears to be the functional homologue of GCD10; however, the two show very little primary sequence homology. In addition, we are preparing antibodies against p66 to be used in immunofluorescence experiments aimed at determining whether p66, like GCD10, is localized in the nucleus.

After identifying the minimal fragment of GCD10 required for RNA binding in vitro, we will analyze the in vivo effects of deletions in this domain. Assuming that the RRM-like sequence is required for RNA binding in vitro, we will also examine effects of point mutations in the conserved RNP-1 and RNP-2 elements on RNA-binding in vitro and *GCD10* function in vivo. For all these experiments, the HA-tagged *GCD10* allele will be mutagenized and introduced into yeast in place of wild-type *GCD10* by plasmid shuffling. We will determine whether the *gcd10* mutations reduce growth rate or lead to derepression of *GCN4* (Gcd$^-$ phenotype). If the mutations are lethal, they will be tested for a dominant slow-growth or Gcd$^-$ phenotype. If a dominant-negative phenotype is observed, we will determine whether the mutant proteins compete with wild-type GCD10 for incorporation into eIF-3 by introducing the mutant HA-tagged *GCD10* allele into the strain containing His-tagged PRT1. Ni-affinity chromatography will be used to purify the eIF3 complex, which will be probed with anti-HA antibodies to detect the mutant *GCD10* protein. A dominant-negative allele could be used in the future to isolate mutations that abolish this phenotype without reducing the level of the mutant protein as a means of identifying residues in GCD10 required for stable complex formation with the other eIF-3 subunits.

We are in the process of cloning the existing *gcd10* chromosomal mutations by plasmid gap-repair and sequencing the rescued alleles. These mutations will be tested for their effects on RNA binding in vitro using the assays described above. It is possible that these mutations affect a functional domain critical for repression of *GCN4* translation that is distinct from that involved in RNA binding, eg. a domain that interacts with eIF-2 or other subunits of eIF3. We will initiate the isolation of additional mutations in *GCD10* by in vitro mutagenesis of the cloned gene. The mutagenized plasmids will be screened in yeast for temperature-sensitive growth on rich medium and for growth on 5-FT plates (*GCN4* derepressed [Gcd$^-$] phenotype) in a strain containing either wild-type chromosomal *GCD10* or *gcd10*Δ.

4. Analysis of GCD10-GCD14 interactions.

The yeast 2-hybrid assay is being used to obtain additional evidence that GCD10 and GCD14 interact with one another, and to identify regions in the two proteins required for these interactions. This approach will be complemented by biochemical analysis of protein-protein interactions using His-tagged GCD10 fusion proteins and GCD14 synthesized by in vitro translation, as described above for analysis of GCD2-GCD7 interactions.

5. Genetic and biochemical identification of other proteins that interact with GCD10 and PRT1.

We are in the process of screening high copy plasmid genomic libraries for suppressors of *prt1* temperature-sensitive mutation, and a P_{GAL1}-cDNA expression library for suppressors of both *gcd10* and *prt1* mutations. This suppressor analysis may identify proteins that specifically interact with these subunits of eIF-3, including other subunits of eIF3 or components of other initiation factors that interact with GCD10 or PRT1. If we do not obtain any new suppressor genes in these approaches, we will turn to the isolation of synthetic lethal mutations that render *gcd10* or *prt1* mutants inviable at a permissive or semi-permissive temperature.

In an alternative approach, we will obtain peptide sequences of any proteins that co-purify with His-tagged PRT1 and His-tagged GCD10 by Ni-affinity chromatography that have not been identified previously in John Hershey's lab at U.C.-Davis. We will avoid using high ionic strength buffers in the purification protocols in an effort to preserve relatively weak interactions, such as those recently detected between GCD14 and the eIF3 complex. From peptide sequences of co-purifying proteins, the corresponding genes will be identified in the yeast genome database. We are particularly interested in obtaining the sequence of a 34 kDa peptide that in preliminary experiments co-purifies with His-PRT1.

To determine the role of GCD10 in maintaining the integrity of the eIF3 complex, we will compare the profiles of proteins that co-purify with His-tagged PRT1 from a wildtype strain and a *gcd10Δ* strain overexpressing $tRNA_i^{Met}$.

6. Analysis of interactions between p39 and other subunits of eIF3.

TIF34 encodes the 39 kDa subunit of eIF3, and contains the WD-40 repeat structure described for the β subunit of the heterotrimeric G-protein transducin. The crystal structure of β-transducin reveals a barrel-like structure in which the repeats comprise the sides of the barrel. It has been suggested that different repeats mediate interactions with different effector molecules. We are considering the possibility that p39 constitutes the structural core of eIF3 and that different subunits interact with different WD-40 repeats in p39. To pursue this idea, we are using the 2-hybrid system to test for interactions between various segments of p39 with PRT1, GCD10, NIP1 and GCD14. To provide a genetic approach to the analysis of p39 interactions, we are carrying out in vitro mutagenesis of the *TIF34* gene in order to isolate temperature-sensitive mutations. These mutations will allow us to isolate dosage and chromosomal suppressors.

D. Regulation of protein kinase GCN2

1. Analysis of the N-terminal regulatory domain and autophosphorylation sites.

GCN2 has a degenerate kinase catalytic domain of ca. 300 residues, located N-terminal to the authentic kinase domain, that contains consensus copies of kinase subdomains VII-XII in the correct order. N-terminal to this pseudokinase domain (ΨPK) is an ca. 80-amino acid segment rich in positively and negatively charged residues, that is probably located on the surface of the protein and may function as a binding site for an effector protein. Both of these features are conserved in the Neurospora homologue of GCN2. We are taking several genetic approaches to address the importance of the ΨPK domain. In one approach, we are using site-directed mutagenesis to substitute conserved amino acids in ΨPK subdomains VII-XII with alanines. We will also construct a chimeric protein in which the yeast ΨPK domain is replaced with that from the Neurospora homologue. If this chimera is functional, it will imply that the exact set of invariant residues is not critically important for ΨPK function. We will also make deletions in the 80-residue N-terminal charged segment and replace it with the corresponding segment from the Neurospora protein. We will determine whether these mutations affect *GCN2* regulatory function in vivo by testing for complementation of *gcn2Δ*, and analyze their effects on autophosphorylation in immune complex assays and on phosphorylation of exogenous eIF2α in whole cell extracts. Results obtained thus far suggest that both the ΨPK and the highly charged regions are required for high-level GCN2 kinase function in vivo and in vitro. In an effort to identify a regulatory protein that might interact with the ΨPK domain, we are planning to use it as bait in a two-hybrid screen of a yeast genomic library.

2. Role of dimerization by GCN2 in activation of kinase function.

There is genetic and biochemical evidence that oligomerization of GCN2 is important for its activation or catalytic function. We found that the slow-growth phenotype of *GCN2*^{c} alleles can be diminished by overexpression of wild-type *GCN2* and completely eliminated by overexpression of the catalytically inactive *gcn2-K559V* protein (dominant-negative phenotype). These observations could indicate that hyperactivated mutant and wild-type or defective GCN2 proteins form heterodimers that are less active than the corresponding GCN2^c homodimers. In addition, we observed two-hybrid interactions between the C-terminal domain of GCN2 with itself, and between the protein kinase domain and itself. It is also noteworthy that the HisRS-like domain of GCN2 contains a good match to motif I, a sequence conserved in class II tRNA synthetases that is involved in dimer formation. It has been proposed that a GCN2 homodimer would bind to a single tRNA molecule and that intermolecular autophosphorylation by the GCN2 protomers in this dimer would activate eIF-2α kinase activity. Consistent with this idea, we found that an internal deletion in the HisRS-like domain of *GCN2* increases its ability relative to wild-type GCN2 to interfere with the functions of *GCN2^c* alleles.

We are now testing deletions in different parts of *GCN2* for their effects on the ability of the wild-type and *gcn2-K559V* proteins to suppress the phenotypes of *GCN2^c* alleles. We wish to determine whether the deletions in the C-terminal and PK domains that abolish the two-hybrid self-association by these regions will eliminate the dominant-negative phenotype of the *gcn2-K559V* protein, as would expected if these regions are required for dimerization by the full-length protein. We will also analyze point mutations which alter the conserved residues in motifs 1-3 of the HisRS-related domain for a dominant-negative phenotype. It has been shown that mutating motif 2 eliminates *GCN2* kinase function in vivo and tRNA binding in vitro to the HisRS-like domain; thus, this mutation may impair tRNA binding to a heterodimer containing one wild-type and one mutated protomer and have a dominant-negative phenotype. A motif 1 mutation might disrupt dimerization of the HisRS domains when present in only one protomer, and thus abolish the dominant-negative phenotype of the *gcn2-K559V* allele.

We have preliminary biochemical evidence for dimer formation by GCN2 from co-immunoprecipitations of an N-terminally truncated form of GCN2 lacking the charged region and ΨPK domain with full-length GCN2 using antibodies that fail to recognize the truncated mutant protein. In addition, we have observed co-immunoprecipitation of full-length GCN2 with a lexA fusion containing the C-terminal domain of GCN2 using anti-lexA antibodies. These biochemical assays provide us with a third means, in addition to the two-hybrid assay and analysis of dominant-negative phenotypes, of testing *GCN2* mutations for their effects on dimerization.

3. Role of homo- and hetero-domain interactions in regulating GCN2 activity.

The two-hybrid analysis indicates that the C-terminal and protein kinase domains of GCN2 interact both with one another and with themselves. This finding led us to hypothesize that binding of the C-terminal domain of GCN2 to its protein kinase domain is involved in inhibiting kinase activity, and that self-association by these domains is mutually exclusive with the interdomain interactions. To pursue this possibility, we will randomly mutagenize the C-terminal domain in the two-hybrid construct and screen for point mutations that weaken its interaction with the protein kinase domain without affecting self-association. Similarly, we will mutagenize the 2-hybrid construct containing the kinase domain and screen for mutations that specifically impair its interaction with the C-terminal segment. Any mutations with the desired phenotypes will be analyzed for their effects on *GCN2* function in vivo after being introduced into full-length *GCN2*.

4. Role of the GCN20 ATP-binding cassettes in ribosome association of GCN1, GCN20 and GCN2, and in activation of GCN2 kinase function.

A C-terminal truncation of *GCN20* that removes both ABC domains is only partially defective for activation of *GCN4* expression under amino acid starvation conditions, indicating that these regions are not essential for GCN20 regulatory function. It is conceivable that the C-terminal truncation of GCN20 alters the conformation of GCN1 in a way that mimicks the active ATP-bound state of GCN20, allowing for activation of GCN2 and derepression of *GCN4* translation at lower levels than occur in wild-type cells. Based on genetic analysis of other ABC proteins, mutations at Gly-237 and Lys-238 in the first ABC domain and at Gly-570 and Lys-571 in the second ABC domain of GCN20 should impair ATP hydrolysis without greatly affecting ATP binding, whereas mutations at Gly-371 and Gly-654 should impair ATP binding and hydrolysis. According to the hypothesis that the ATP-bound form of GCN20 promotes activation of GCN2 kinase function in amino acid-starved cells, these two sets of mutations should have opposite effects on *GCN4* expression: A GCN20 protein defective for ATP binding would be locked into an inactive configuration, impairing GCN2 activation in response to starvation to the same extent seen for a complete deletion of *GCN20*. In contrast, mutations that prevent ATP hydrolysis but not ATP binding might lock GCN20 into the active form that permits stimulation of GCN2 kinase activity when uncharged tRNA accumulates in amino acid-starved cells, the same effect postulated above for deletion of the ABC domain.

An alternative explanation for the modest phenotypic effect of deleting the ABC domains of *GCN20* is that a close homologue of *GCN20* (identified in the yeast sequencing project) can substitute for GCN20 in stimulating GCN2 activation in conjunction with GCN1. We will test this possibility by deleting the *GCN20*-homologous gene in strains containing wild-type *GCN20*, *gcn20Δ*, or a truncation of *GCN20* that removes the ABC domains, and analyze *GCN4* expression in the resulting mutant strains. If the presence of the *GCN20* homologue does not account for the modest effect of deleting the GCN20 ABC domains, it could be proposed that these sequences function to control the extent of GCN2 activation or insure that it occurs only during sustained amino acid limitation when uncharged tRNA binds to the ribosomal A site at high frequencies. In the absence of the ABC domains, activation of GCN2 might then occur inappropriately under conditions of transitory or limited starvation that would not normally activate GCN2. It is even possible that the ABC domains provide a means of preventing GCN2 activation by uncharged tRNA under starvation conditions where an essential nutrient like carbon or nitrogen is missing, or under stress conditions, where the derepression of amino acid biosynthetic pathways may not be productive. When the ABCs of GCN20 are deleted, GCN2 would still be activated by uncharged tRNA; however, the ability to override activation of GCN2 in response to starvation for essential nutrients besides amino acids would be lost.

To test these different ideas, the point mutations in the ABC domains of GCN20 described above will be analyzed for their effects on *GCN4-lacZ* expression under nonstarvation conditions, and in response to different degrees of amino acid deprivation. In addition, we will determine the kinetics of repressing *GCN4-lacZ* expression following a return to amino acid sufficiency. We will investigate the effects of carbon, nitrogen or sulfur starvation on GCN4-lacZ expression, and the effects of these limitations on the derepression response to amino acid starvation. If the point mutations in the GCN20 ABCs have interesting phenotypes, we will explore the possibility that they affect complex formation between GCN1 and GCN20 as assayed by co-immunoprecipitation.

We found that ribosome-association of GCN20 is strongly dependent on GCN1, whereas GCN20 is dispensable for the ribosome-association of GCN1. The amount of both proteins found associated with ribosomes is stimulated by ATP in the gradients; however, deletion of *GCN20* does not decrease the ribosome-association of GCN1. It is possible that the GCN20 homologue mentioned above can substitute for GCN20 in stimulating ribosome association by GCN1, and this possibility will be tested once the homologous gene is deleted in wild-type and *gcn20Δ* backgrounds. In any case, we will analyze the deletions and point mutations in conserved residues of the GCN20 ABC domains for their effects on ribosome association of GCN20 and GCN1 and for the stimulatory effect of ATP on ribosome binding by these two proteins.

It has not been possible thus far to detect a physical interaction between GCN2 and GCN1/GCN20 by co-immunoprecipitation experiments under conditions where the GCN1/GCN20 complex is readily demonstrable. As an alternative means of establishing a physical link between these proteins, we will investigate whether GCN1 and GCN20 are involved in recruiting GCN2 to ribosomes by examining the effects of deletion mutations in *GCN1* and *GCN20* on the polysome association of GCN2. ATP will be added to the gradients to maximize the binding of GCN1 and GCN20 to the ribosome. The effects of deleting *GCN1* and *GCN20* on GCN2 binding to ribosomes will be examined first, followed by an analysis of point mutations in conserved residues of the ABC domains of GCN20 to determinine whether impairing the ability of full-length GCN20 to bind or hydrolyze ATP alters the ribosome association of GCN2.

We will also employ the yeast two-hybrid assay in an effort to detect interactions between particular segments of GCN2 with either GCN1 or GCN20. In a complementary biochemical approach, we will produce in bacteria GST fusion proteins containing various domains of GCN2, or a full-length GCN2-GST fusion, add them to whole cell yeast extracts, and analyze the proteins that co-purify with the fusion proteins on glutathione affinity columns. By adding GST-GCN2 fusion proteins to the extracts at much higher concentrations than that of wild-type GCN2, we may detect a weak interaction between GCN2 and the GCN1/GCN20 complex that would not be stable enough to survive the washing steps in immunoprecipitation experiments. We will also express a His-tagged form of GCN2 and examine the profile of proteins that co-purify with the tagged protein on Ni-affinity columns. Any proteins that specifically co-purify with tagged GCN2 will be analyzed by partial peptide sequencing to identify the corresponding structural genes in the yeast genome database.

5. Examination of purified GCN20 and GCN1 for ATPase, GCN2- and RNA-binding activities

Epitope-tagged GCN1 and GCN20 constructs will be used to assay immunoprecipitates containing these proteins for binding and hydrolysis of ^{32}P-ATP and ^{32} P-GTP. If this approach is successful, we will analyze the effects of mutations in the ABC domains of GCN20 on these activities. As the ATPase and GTPase activities of EF-3 are dependent on the presence of yeast ribosomes, detecting GCN20 NTPase activity may require the addition of purified yeast ribosomes. If this approach fails, we will employ His-tagged GCN20 proteins expressed in yeast to purify the GCN1/GCN20 complex and assay it for ATPase and GTPase activities. If successful, the effects of mutations in the GCN20 ABC domains on these activities will be addressed. His-tagged mutant and wild-type GCN20 expressed in bacteria also will be assayed for NTPase activity independently of GCN1. If NTPase activity that is dependent on the GCN20 ABC domains is detected by one of these approaches, we will determine whether it is stimulated by uncharged tRNA (either free or bound to the A site of ribosomes). We will also test purified His-tagged GCN20 for the ability to bind purified rRNA or tRNA (ref RNA binding assay) and for the ability to bind GCN2 in yeast whole cell extracts. A poly-histidine tagged form of GCN1 will also be purified from bacteria and tested for tRNA-, rRNA- and GCN2-binding activities. If any binding activities are detected for GCN1 or GCN20, we will attempt to identify a specific domain responsible for the activity by assaying internal deletions and point mutations in the relevant protein. If any other proteins co-purify from yeast cells with His-tagged GCN1 or GCN20, we will obtain limited peptide sequence information from the gel-purified polypeptide to identify the corresponding gene in the yeast genome database.

6. In vitro reconstitution of activation of GCN2 by uncharged tRNA on translating ribosomes.

Thus far, we have been unable to activate GCN2 kinase function in vitro with uncharged tRNA. Having now shown that GCN1 and GCN20 are ribosome-associated, we will attempt to reconstitute activation of GCN2 by uncharged tRNA in vitro using polysome-containing fractions from sucrose gradients enriched for GCN1/GCN20 and GCN2. We will determine whether GCN2 kinase activity in these fractions can be stimulated by uncharged yeast tRNA, and if so, whether this activation is abolished by point mutations in the HisRS domain that decrease tRNA binding to GCN2 in vitro. We will also investigate the effects on GCN2 in vitro kinase activity of starving for amino acids prior to extract preparation, of mutations that lead to constitutive activation of kinase activity (*GCN2*^{c} alleles) and of internal deletions or point mutations in *GCN1* and *GCN20* that impair activation of GCN2 in amino acid starved cells.

7. Identify novel proteins that interact with GCN1.

Since GCN1 can bind to polysomes in the absence of GCN20, it would be very informative to identify the ribosomal proteins that GCN1 interacts with and characterize their binding domains. Two different genetic approaches will be taken to identify ribosomal proteins that interact with GCN1. One approach will involve screening of yeast genomic and cDNA libraries by the two-hybrid technique using different segments of GCN1 as the bait. In a second approach, we will attempt to isolate high-copy or chromosomal suppressors of the slow-growth phenotype associated with overexpressing GCN1.

8. Genetic screening for novel proteins that interact with GCN2.

The location of GCN2 on the ribosome and possible roles of specific ribosomal proteins in this regulation are unknown, as is the mechanism for coupling GCN2 kinase activity to purine levels. It is unlikely that the *gcn* class of mutations has been saturated, and there may be additional proteins besides GCN1 and GCN20 involved in controlling GCN2 activity. For these reasons, we will pursue several genetic approaches aimed at the identification of proteins that interact with GCN2. In one approach, we will use yeast two-hybrid constructs containing different segments of GCN2 to screen the Elledge cDNA library and the Clontech genomic library. We have initiated this approach using a bait construct containing the C-terminal ribosome binding domain of GCN2, and we will proceed to one containing the ΦPK domain.

In a second approach, we are attempting to saturate the *gcn* class of mutations by random insertional mutagenesis of the yeast genome using the insertion library constructed in Michael Snyder's lab. This is a plasmid library of genomic fragments containing Tn3::*lacZ*::*LEU2* transposons inserted at random sites. The mutagenized yeast fragments are removed from the plasmid vector by digesting with NotI and then used to make chromosomal insertions by transforming a yeast strain to Leu$^+$. Using this library, we have mutagenized a haploid strain that is wild-type for all known *GCN* and *GCD* genes (except for *GCN4*) and contains an integrated *HIS3-gus* fusion under the control of GCN4 and an integrated *GCN4-lacZ* fusion. The strain carries a *gcn4* allele that contains only the N-terminal activation domain, which confers high-level activation of GCN4-regulated genes under standard starvation conditions but is defective relative to wild-type *GCN4* under more severe starvation conditions. By incorporating this attenuated allele in place of wild-type *GCN4,* we hope to increase the sensitivity of our screen for *gcn* mutations. (In addition, we may identify mutations in nonessential transcriptional co-activator proteins, such as *GCN5*, that impair transcriptional activation mediated by the N-terminal activation domain; see below). Transformants containing insertions in different loci are screened for increased sensitivity to 3-AT, sulfometuron (inhibits Ile and Val synthesis), and azaadenine (azA) (inhibits purine biosynthesis), indicating a Gcn$^-$ phenotype.

Transformants showing sensitivity to 3-AT and SM or to azA are subjected to quantitative assays of the two integrated fusions after growth in the presence of 3-AT or aZA and ranked according to the magnitude of their derepression defects. (Note that 1/6 of the chromosomal insertions will fuse *lacZ* in-frame to the disrupted gene. For this class of insertion mutants, we will conduct Western analysis of *GCN4* expression using antibodies against GCN4.) Any mutants showing reduced *GCN4* expression under starvation conditions will be analyzed genetically to determine whether this phenotype results from a single insertion. Mutants showing reduced *HIS3-gus* expression but normal *GCN4* expression are candidates for GCN4 transcriptional co-activators (see below). For mutants of interest, we are isolating the genomic sequences adjacent to the insertion site using a plasmid integration-excision scheme and determining the sequence of 100-200 nt. With this information we can identify the corresponding gene in the yeast database. From the results obtained thus far, we have identified several novel genes that appear to be required for transcriptional activation of *HIS3* but not for derepression of *GCN4* expression, phenotypes indicative of a transcriptional co-activator.

E. Molecular mechanism of transcriptional activation by GCN4

1. Biochemical analysis of potential interactions between GCN4 and transcriptional mediators.

Our mutational analysis of GCN4 has identified 7 clusters of 2-3 aromatic or bulky hydrophobic amino acids that make redundant contributions to transcriptional activation by GCN4. Some or all of these residues could mediate important interactions between GCN4 and its target proteins in the transcriptional machinery. Genetic analysis has implicated the ADA2, ADA3 and GCN5 proteins as mediators of transcriptional activation by GCN4 and several other acidic activators; however, until now there has been no evidence that GCN4 physically interacts with these proteins or with any of the general transcription factors. We are using the point mutations we generated in the activation domain of GCN4 to identify target proteins in the transcription machinery and to identify the domains in these proteins that contact GCN4.

We have been testing GCN4-GST fusion proteins for interactions with candidate transcription factors using a panel of GCN4-GST fusion proteins overexpressed in *E. coli*, including wild-type GCN4 and mutants containing alanine substitutions in the 7 critical hydrophobic clusters in the activation domain. Bacterial extracts containing equal amounts of these fusion proteins are mixed with aliquots of whole cell extracts from yeast strains expressing HA-tagged forms of various transcription factors. After isolating the GST-GCN4 proteins on glutathione-agarose beads (GST pull-down assay), the immobilized proteins are probed by immunoblotting with anti-HA antibodies to detect recovery of the HA-tagged proteins. Using this approach, we have detected specific interactions between GCN4 and two TBP-associated factors, $TAF_{II}60$ and $TAF_{II}90$, suggesting that recruitment of TFIID to the promoter is one means whereby the GCN4 activation domain stimulates transcription by RNA Polymerase II. Additional experiments will be carried out to determine which of the 7 clusters of critical hydrophobic residues in the GCN4 activation domain is responsible for its interaction with $TAF_{II}60$ and $TAF_{II}90$ in yeast extracts. In addition, we will analyze other TAF proteins for interaction with GCN4 to determine whether a complex containing all the known components of TFIID is the $TAF_{II}60/TAF_{II}90$-containing entity in the extracts that interacts with GCN4. In another set of experiments we will test isolated TAFs, beginning with $TAF_{II}60$ and $TAF_{II}90$, expressed from baculovirus vectors and purified from insect cells, or synthesized by in vitro translation in rabbit reticulocyte lysates, for the ability to interact specifically with GCN4. These latter studies will enable us to determine whether GCN4 interacts with particular TAFs directly, or whether these interactions are mediated by other factors in the yeast extract.

We have recently detected a specific interaction between GST-GCN4 fusion proteins and an HA-tagged form of ADA2 in yeast cell extracts using the same GST pull-down technique described above. We will now test for interactions between the GST-GCN4 fusion proteins and HA-tagged ADA3 and GCN5 to determine whether the entire ADA complex is interacting with GCN4, and probe for interactions between the GST-GCN4 fusions and ADA2 expressed alone. We also detected a specific interaction between the SWI3 protein and the GST-GCN4 fusion protein in yeast cell extracts. SWI3 is a subunit of the SNF/SWI complex that is thought to facilitate activation of certain yeast genes by remodeling chromatin structure. The interaction between SWI3 and GST-GCN4 was not diminished by mutations in the critical hydrophobic residues in the GCN4 activation domain. It is possible, however, that GCN4 interacts with SWI3 via its DNA-binding domain. To pursue these observations, we will first determine whether deletion of *SWI3* impairs activation of GCN4 target promoters in vivo. If so, we will investigate the binding of GST-GCN4 fusions to other components of the SWI/SNF complex in yeast extracts, examine interactions between GCN4 and purified SWI3, and attempt to identify specific residues in GCN4 that mediate its interaction with SWI3.

Using a complementary genetic approach, we will attempt to isolate mutations in one or more of the genes encoding TAF proteins that affect transcriptional activation by GCN4 in vivo. We will begin by in vitro mutagenesis of plasmids bearing the $TAF_{II}60$ and $TAF_{II}90$ genes and screen pools of mutagenized plasmids in yeast strains deleted for the chromosomal TAF-encoding genes for defects in activation by GCN4. Defects in GCN4 function can be easily recognized by increased sensitivity to various amino acid analogues and reduced expression of *lacZ* reporters fused to GCN4 target promoters (eg. *HIS3).* The mutations will be sequenced to identify specific residues in the TAF proteins that are critical for activation by GCN4. TAF complexes bearing the mutant proteins will be tested for altered interactions with GST-GCN4 proteins in whole cell extracts. In addition, we will investigate whether GCN4-dependent transcription in vitro is affected by the TAF mutations. This mutational analysis will provide in vivo evidence that the TAF proteins are targets of the GCN4 activation domain and identify points of contact between GCN4 and individual TAF proteins.

2. Genetic screens for transcriptional factors that interact with GCN4.

a. Suppressors of a leaky *GCN4* allele with point mutations in the activation domains.

We are isolating chromosomal suppressors of the 3-AT-sensitive phenotype of the *gcn4-1843* allele, which contains alanine substitutions in three of the critical hydrophobic clusters in the activation domain (Met-107/ Tyr-110/ Leu-113, Trp120/ Leu-123/Phe-124, and Phe-97/Phe-98). Assuming that these mutations weaken interactions between GCN4 and one or more co-activator proteins or basic transcription factors, we anticipate that chromosomal mutations can be isolated in these factors that will restore their interaction with mutant GCN4. By testing these mutations for the ability to suppress other *gcn4* alleles that contain a different set of point mutations, we may identify suppressors that specifically restore interactions with particular activation modules of GCN4. We may also isolate mutations in other components of the transcription machinery which bypass the requirements for those activation modules which are lacking in the *gcn4* allele. It is conceivable that certain transcription factors that are normally recruited or activated by GCN4 could be mutated to mimic the effect on them that wild-type GCN4 normally exerts. Even if they map in known proteins, such bypass mutations would highlight the importance of these proteins in transcriptional activation by GCN4. Of a large number of revertants analyzed thus far, we have obtained only second-site suppressors in *GCN4* instead of unlinked *trans*-acting suppressors. One final group of revertants will be analyzed and if no unlinked suppressors are present in this group, we will abandon this approach. It may be interesting, however, to characterize the second-site mutations in *GCN4* to detemine what amino acid changes can that restore the activation function of this defective *gcn4* protein.

F. Transcriptional repression of *ADE* genes by down-regulation of *BAS1* and *BAS2*.

Our results showing *BAS2*-dependent, adenine-repressible transcriptional activation of a *lexA* reporter by a lexA-BAS1 fusion strongly suggests that complex formation by BAS1 and BAS2 is required to constitute a potent activator at the promoters of *ADE* genes. The fact that activation by both authentic BAS1 and the lexA-BAS1 fusion is stimulated under adenine-repressing conditions by overexpression of BAS2 indicates that BAS2 is limiting for activation under repressing conditions and suggests that down-regulating complex formation by BAS1 and BAS2 is an important aspect of adenine repression. The result that deleting the C-terminal 22% of BAS1 from the lexA-BAS1 fusion protein led to constitutive high-level activation of the *lexA* promoter that was independent of *BAS2* strongly suggests that BAS1 contains a latent activation domain that is functionally masked by the C-terminal segment under adenine repressing conditions, and that complex formation with BAS2 antagonizes the negative-acting domain and unmasks BAS1 activation function.

We will carry out an extensive mutational analysis of the functional domains in BAS1 and lexA-BAS1 that are required for BAS2-dependent transcriptional activation. We wish to map precisely the boundaries of the activation domain in BAS1, the negative-acting domain in BAS1 that mediates adenine repression of its activation function, and the points of contact between BAS1 and BAS2 that are responsible for unmasking the activation domain of BAS1. We will begin by making small internal deletions of ca. 25 amino acids across the coding region of the BAS1-lexA fusion, and follow up with clustered substitution mutations in the critcal intervals. We will also attempt to isolate additional *BAS1^c* mutations that resemble *BAS1^c-D617N* in allowing BAS1 to activate transcription under repressing conditions in a manner that is fully BAS2-dependent. Such mutations may overcome the repressive effect of adenine on BAS1-BAS2 complex formation and, thus, identify critical regulatory regions in BAS1. We will also attempt to isolate *BAS2* mutations with the same phenotype.

We are working to develop a co-immunoprecipitation assay for BAS1-BAS2 complex formation that could be used to examine the effects of mutations on complex formation in vivo. We will also investigate the use of His-tagged or GST-tagged forms of these proteins expressed in *E. coli* to reconstitute and analyze the requirements for complex formation in vitro. In addition, we are investigating the possibility that nuclear localization of BAS1 is negatively regulated by adenine in the medium using immunofluorescence of lexA and HA-tagged forms of BAS1.

Publications:

1. Grant CM, Miller PF, Hinnebusch AG. Sequences 5' of the first upstream open reading frame in *GCN4* mRNA are required for efficient translational reinitiation, Nuc Acids Res, 1995;23:3980-8.

2. Hinnebusch AG. Translational control of *GCN4:* gene-specific regulation by phosphorylation of eIF2. In: Hershey JWB, Mathews MB, Sonenberg N, eds. Translation control. New York: Cold Spring Harbor Laboratory Press, 1996;199-244.

3. Jackson BM, Drysdale CM, Natarajan K, Hinnebusch AG. Identification of seven hydrophobic clusters in GCN4 making redundant contributions to transcriptional activation. Mol Cell Biol, in press.

4. Taylor DR, Lee SB, Romano PR, Marshak DR, Hinnebusch AG, Esteban M, Matthews MB. Autophosphorylation sites participate in the activation of the double-stranded RNA-activated protein kinase PKR. Mol Cell Biol, in press.

5. Yang W, Hinnebusch AG. Identification of a regulatory subcomplex in the guanine nucleotide exchange factor eIF2B that mediates inhibition by phosphorylated eIF2. Mol Cell Biol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01009-04 LEGR

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation and function of retroelements

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: H.L. Levin, Senior Staff Fellow, (All listed personnel LEGR:NICHD)

A. Atwood	Biologist
D. Balasundarum	EBON Fellow
V.D. Dang	Vis. Fellow
J.H. Lin	IRTA Fellow
J.K. Choi	Pre-IRTA Fellow

COOPERATING UNITS *(if any)*

Dr. Jef Boeke, Johns Hopkins University, Baltimore, MD
Dr. Michael Benedik, University of Houston, TX

LAB/BRANCH

Laboratory of Eukaryotic Gene Regulation

SECTION

Unit on Eukaryotic Transposable Elements

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.1	4.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our research program focuses on the mechanisms of retroelement action. To understand the complex functions required for the propagation of retroelements we study retrotransposons, a family of elements that are closely related to retroviruses. A significant advantage of studying retrotransposons is that they exist in hosts such as yeast that can readily be studied using sophisticated molecular genetic techniques. In the process of characterizing yeast transposition, we have obtained strong evidence that Tf1 reverse transcriptase uses a novel mechanism of self-priming to initiate cDNA synthesis. This is in contrast to the tRNA mechanisms thought to be used by all other LTR-containing elements. The first 11 bases of the Tf1 transcript anneal to the primer binding site in a process that results in the initiation of reverse transcription. Primer extension assays and splinted ligation reactions were used to identify substantial amounts of the Tf1 transcript that were cleaved between the 11th and 12th bases. These results support the model that the first 11 bases of the Tf1 mRNA must be cleaved from the transcript to provide a 3'OH to serve as a primer for DNA synthesis. Mutations in the RNase H of Tf1 caused a sharp reduction in the accumulation of the cleaved product indicating that Tf1 RNase H may possess an unusual ability to recognize and cleave an RNA duplex. The overall importance of the self-priming mechanism was underscored by recent sequence analyses that indicated 7 other retroelements isolated from a variety of filamentous fungi probably utilize this self-priming mechanism. In each case strong complementarity exists between the primer binding site and the 5' region of the mRNA. The examination of the 5' untranslated region of the Tf1 mRNA revealed that this sequence possesses the potential to form a complex RNA structure composed of 39 basepairs that includes the primer binding site. This region of Tf1 was subjected to extensive mutagenesis that revealed that much of the structure contributes to transposition. The majority of the mutations in the structure resulted in a substantial reduction in the levels of cDNA produced. Second-site mutations that restored the complementarity of the structure resulted in elevated transposition frequencies and cDNA levels. These compensating mutations demonstrated that formation of the RNA structure is required for transposition and reverse transcription. Further analysis of the Tf1 RNA produced by elements with mutations in the RNA structure revealed that formation of the structure was required for the cleavage reaction that generated the primer. To characterize the Tf1 encoded factors required specifically for integration, we have screened a large set of mutant elements to identify those that are defective for transposition but nevertheless produce high levels of cDNA. Thus far, we have identified 7 mutations that reduce integration. Interestingly, two were found to be in RNase H, an enzyme thought to be required only for reverse transcription. Quantitative analysis indicated these mutations appear to have no effect on the levels of cDNA that accumulate. These results suggest that RNase H may provide a function that is directly required for integration.

Project Description

Objectives

Our efforts are designed to elucidate the molecular basis of each step in the process of retroelement proliferation. The most medically important class of these elements are the retroviruses, members of which are responsible for AIDS as well as several types of cancer caused by the proviral induction of oncogene expression. Many aspects of particle assembly, reverse transcription, nuclear entry and chromosome integration are difficult to study because retrovirus hosts possess a high level of genetic complexity. Our approach to understanding these events is to study retrotransposons, a family of elements that are closely related to retroviruses. A significant advantage of studying retrotransposons is they exist in yeast, a host that can be studied readily using sophisticated molecular genetic techniques. The similarity of retrotransposons to retroviruses includes the presence of two long terminal repeats (LTRs) as well as open reading frames (ORFs) with coding sequences homologous to retroviral protease (PR), reverse transcriptase (RT), and integrase (IN). The first step in the transposition pathway is synthesis of a full-length mRNA with sequence that begins in the 5' LTR and terminates in the 3' LTR. This is directly analogous to the initial step in retrovirus particle formation. Retroviral and retrotransposon mRNAs are translated into proteins that assemble along with the mRNA into large particle structures. Both retroviral and retrotransposon particles undergo a maturation process that includes the proteolytic processing of precursor proteins and the reverse transcription of the mRNA. Retroviral particles are able to escape the host cell and integrate their DNA into the genome of neighboring cells, while retrotransposon particles complete transposition by simply inserting their DNA into the genome of the original host cell. Because each step in retrotransposition is directly related to a retrovirus process, results from the investigation of yeast retrotransposition will be relevant to aspects of retrovirus behavior.

The retrotransposon we study is the Tf1 element of the fission yeast, *Schizosaccharomyces pombe*. The transposon is 5 kb and contains a single ORF of 1340 amino acids with coding sequences homologous to PR, RT, and IN. We have previously demonstrated, using an *in vivo* assay, that Tf1 is active and can transpose at a significant frequency. The system we have developed allows us to use a battery of powerful molecular and genetic techniques to identify and characterize factors that contribute to the transposition process.

1. The identification of host factors that contribute to transposition.

One of our primary interests is the interaction between the host cell proteins and the transposon because it is this that is the least understood in the retrovirus systems. Genetic experiments on retroviruses have defined the principal role of each of the major virus proteins by altering the virus encoded factors and assaying for an effect on virus functions. Studying retroelements in yeast allows us to explore the contributions of the host proteins to the entire process of transposition.

The genetic approach we are using to identify proteins that function in transposition is to use a version of the transposition assay to screen individual colonies for mutations that reduce transposition. We developed a genetic screen for transposition that depends on the overexpression of Tf1 transcripts containing a selectable marker that is reverse transcribed during transposition and inserted into the host genome. This allowed us to develop a screen of large numbers of colonies for host mutations that alter the transposition frequency.

2. The essential functions of transposon proteins.

Another goal associated with the mechanisms of transposition is to learn which parts of the Tf1 proteins are required for activity and what are their roles in the process. Towards this goal, we have assembled a large set of random mutations in Tf1 that block transposition. By characterizing the properties of these mutations, we hope to associate the domains of each protein with activities required for specific mechanisms in the transposition pathway.

3. The self-priming of Tf1 reverse transcription.

A unique aspect of Tf1 is that its sequence contains no homology to tRNA molecules at the primer binding site (PBS), the site where reverse transcription is initiated. This is in direct contrast to all other LTR-retroelements which have 15-20 bases of perfect homology to specific tRNA molecules that serve as primers for reverse transcriptase during the first step of cDNA synthesis. Recent results indicate that Tf1 uses a novel self-priming mechanism of reverse transcription that defines a new family of retroelements. The first 11 bases of the Tf1 transcript anneal to the primer binding site in a process that results in the initiation of reverse transcription. Our goal is to understand how the formation of this RNA duplex results in the priming of reverse transcription.

Methods Employed

We have used a wide variety of molecular, genetic, and biochemical techniques in experiments described in this report. Our genetic approaches include several standard yeast techniques such as transformation, gene replacement, mating, tetrad dissection, diploid formation and mutagenesis. We are also using the two-hybrid system as a general method for identifying interactions with Tf1 proteins.

Our standard transposition assays take advantage of two drug marker selections. We use 5-fluoroorotic acid to select for cells that lose plasmid copies of the URA3 gene. Our transposons are marked with the bacterial *neo* gene that provides *S. pombe* with resistance to high concentrations of G418. We have also started to use an artificial *S. pombe* intron placed within marker genes in the transposon to assay for reverse transcription events.

Biochemical methods include the use of various yeast expression systems to improve production of the Tf1 proteins. We now use the *nmt1* promoter of *S. pombe* to regulate levels of protein synthesis. The Tf1 ORF is fused to the *nmt1* promoter in a multicopy plasmid. The presence of 10 µM vitamin B1 reduces expression 50-fold. *nmt1* is the strongest *pombe* promoter thus far characterized. Extracts from these expression strains are then subjected to sucrose gradient sedimentation as well as other purification protocols. Fractions from these gradients are immunoprecipitated using antibodies raised against Tf1 proteins.

A full range of molecular biology techniques are utilized to make reagents and characterize them. Constructs are made by using restriction enzymes, phosphatases, DNA polymerases, PCR and ligase. We routinely sequence the DNA of complex constructs. Immunoblots, DNA blots and RNA blots are all used to characterize various strains.

Major Findings:

I. The primer of Tf1 reverse transcription is generated by a cleavage between the 11th and 12th bases of the Tf1 mRNA.

1. Review of the evidence that Tf1 uses an unusual mechanism of self-primed reverse transcription.

The role of tRNA species as the primer of reverse transcription is thought to be common to all LTR-containing retrotransposons and retroviruses because all the elements that have been sequenced have a PBS that is complementary to known tRNA species. In light of the conservation of reverse transcription priming, it is surprising that no tRNA primer has been identified for Tf1. In a recent report, data was presented supporting the model that Tf1 undergoes a novel mechanism of self-primed reverse transcription (Levin 1995). See Figure 1. The observation was made that the first 11 bases of the transcript are complementary to the PBS as identified by previous primer extension studies (Levin, Weaver et al. 1993; Levin 1995). The formation of a looped structure containing this RNA duplex was proposed to lead to the priming of minus-strand strong-stop DNA. Mutations made in the PBS and the 5' end of the Tf1 transcript greatly reduce the *in vivo* transposition activity and the accumulation of minus-strand strong-stop DNA in Tf1 particles (Levin 1995). The combination of single base mutations in the 5' end of the transcript with individual mutations in the PBS that result in restored complementarity, rescue the transposition defects of the individual mutations and therefore demonstrate that the 5' end of the transcript does anneal to the PBS. Because the formation of the loop structure does not directly result in the presentation of a 3'OH that could be used by RT for priming, we speculated that a nuclease may cleave within the RNA duplex and thus create a 3'OH. An alternative possibility is that Tf1 RT may prime minus strong-stop DNA from an internal base at a 2'OH, as is the case for the retron reverse transcriptases of bacteria (Lampson, Sun et al. 1989; Lampson, Inouye et al. 1989; Hsu, Eagle et al. 1992). Little evidence exists that favors one over the other of these two possibilities except that primer extension reactions used to map the 5' end of Tf1 transcripts never showed any evidence of a species of Tf1 mRNA that is cleaved within the first 11 bases (Levin and Boeke 1992; Levin, Weaver et al. 1993). As a result, the function of the folded RNA loop in the mechanism of reverse transcription was unresolved. The importance of this issue is enhanced by the evidence that indicates the self-priming mechanism of reverse transcription defines a new family of retrotransposons that includes two other elements, CfT-I, and Tf2 (Levin 1995).

2. *In vivo* experiments to distinguish between two types of self-priming mechanisms.

To continue our examination of the model that the primer is generated by cleavage of the mRNA, we perused primer extension assays as a means of detecting Tf1 mRNA that lacked sequence from the 5' end. As stated above, RNA extracted from cells induced for Tf1 transposition, showed no evidence of cleavage at the 5' end of the Tf1 mRNA. One complication of this result was that the putative cleavage event may be coupled to reverse transcription so that if transcripts were cleaved, they would have been immediately degraded by RNaseH.

To preserve priming intermediates from degradation, we used site-directed mutagenesis to generate five Tf1 plasmids each with a mutation in an active site residue of the RT polymerase or RNase H. The mutations in the RNase H active site residues would directly inhibit the degradation of Tf1 mRNA where as the mutations in the DNA polymerase domain of RT were expected to protect any cleaved Tf1 transcripts from

RNase H degradation because no DNA:RNA heteroduplex would be produced. Several groups have identified 3 amino acid positions within RNaseH of HIV and moloney leukemia virus that can be mutated to produce an RT with normal polymerase activity and no RNaseH function (Repaske, Hartley et al. 1989; Mizrahi, Brooksbank et al. 1994). Using these studies as a guide, as well as the identification of active site residues from X-ray crystal structures of other RNaseH molecules (Katayanagi, Miyagawa et al. 1990; Yang, Hendrickson et al. 1990; Davies, Hostomska et al. 1991; Kohlstaedt, Wang et al. 1992), we individually changed two essential aspartic acids (710 and 779, numbered from beginning of the orf) to asparagines and alanines. Two critical aspartic acids in the polymerase domain of Tf1 RT are numbers 563 and 564 and were mutated to asparagine.

The effects of the five point mutations generated in RT were first assessed by measuring the transposition frequencies of *S. pombe* strains bearing mutant versions of Tf1 as expressed from the nmt1 promoter on the plasmid pHL449-1. Cells are monitored for transposition using a *neo* gene inserted into the Tf1 in pHL449-1 (Levin 1995). After patches of cells are induced for transcription on plates lacking thiamine and subsequently grown on 5-FOA to select against the presence of pHL449-1, cells that undergo transposition can be detected on G418/5-FOA agar because genomic copies of *neo* accumulate as the result of transposition. Each of the point mutations in Tf1 RT caused a dramatic reduction in transposition frequency, suggesting the conserved aspartic acid residues in Tf1 are critical for RT activity.

To assess directly the impact of the mutations on reverse transcription, the levels of reverse transcripts in cells were measured by DNA blot analysis. Cultures were induced for transposition in liquid medium and total DNA was isolated. As reported previously, levels of mature sized double-stranded cDNA can be determined by digesting total DNA with *Bst* XI and then performing DNA blot analysis (Atwood, Lin et al. 1996). We found that the 2.1 kb cDNA fragment derived from the right end of Tf1-*neo* was produced by the wild-type Tf1 but was absent in all of the strains that carried mutations in RT and the control strain that contained a frameshift in PR. These data indicate the RT mutations inhibited transposition by directly reducing the levels of reverse transcripts.

The ability of the RT mutations to allow the accumulation of Tf1 mRNA cleaved near the 11th base was tested by primer extension analysis of RNA extracted from particles and annealed to a ^{32}P-labeled oligo. The RNA isolated from strains with the mutations in residues 710 and 779 of RNase H showed no evidence of cleavage, while all three mutations in the polymerase domain, D563N, D563L, and D564N, resulted in primer extension products that were approximately 11 bases shorter than full-length. The presence of the shorter extension products suggested that the Tf1 RNA may be cleaved near the 11th base. To determine exactly the size of the shorter extension product, a sample of the extension reaction was run adjacent to the sequence ladder produced with the same oligo. The shorter product comigrated with the G at position 199 of Tf1 which is the 12th base of the native mRNA and is therefore consistent with the possibility that a cleavage occurred between the 11th and 12 base of the Tf1 mRNA.

3. A splinted ligation assay for cleaved Tf1 mRNA.

The primer extension assays suggested that the Tf1 primer was generated by a cleavage of the mRNA but, because of the indirect nature of the assay, the same shortened extension product could also be the result of a base-specific modification that could cause premature termination during the extension assay. Because an alternative mechanism for the priming of Tf1 reverse transcription includes initiating DNA synthesis from a 2'OH, it was important to determine whether the shorter extension product was the result of cleaved RNA or the presence of a 2'OH branch point on the 11th base.

If the Tf1 RNA was cleaved and the first 11 bases functioned as primer, a 3' OH must have been present on the primer, leaving the phosphate attached to the 5' end of the remaining RNA. Therefore, a cleavage could be specifically detected by the ligation of the 3'OH of an oligonucleotide to the 5' end of the large RNA product of the cleavage reaction. The resulting product would be a Tf1 DNA-RNA chimera that could be longer than the full-length message. The ligation of a DNA oligonucleotide to the 5' end of an RNA with a 5' phosphate can be catalyzed by T4 DNA ligase if an additional oligo is used as a splint to position together the 3' and 5' ends of the ligation substrates (Moore and Sharp 1992). Figure 2 is a diagram of the components of the splinted ligation reaction. Because the 3' end of the DNA oligo and the 5' end of the RNA must be splinted without a gap, a set of 5' oligos could be designed to map the exact position of a cleavage if one were detected. This strategy is also diagrammed in Figure 2. Total RNA from a strain carrying the D564N mutation in the YXDD motif, was mixed with T4 DNA ligase, the splint oligo (HL193), and one of each of the 5' oligos. To characterize the products of the ligation reactions, oligo JB890 was used in *in vitro* primer extension reactions of the ligation products to identify RNA molecules that were 6 bases longer than the full-length Tf1 transcript, the size predicted for RNA that ligated to one of the DNA oligos. Only one of the 1-hour ligation reactions resulted in a larger extension product than the full-length band, indicating that the 5' oligo HL197 did ligate to the 5' end of the RNA. The exact size of the extension product templated by the oligo HL197 ligated to the RNA was as predicted, six bases larger than the full-length product. A 3 hour reaction that contained HL197 also produced this ligated product, but in this case, no shorter extension product remained, demonstrating that the entire population of RNA had been quantitatively converted into the ligated product. These results showed that the shorter extension product templated by Tf1 mRNA was the result of cleavage and not the presence of a modification such as a 2'OH linkage. The oligo specificity was maintained in the 3-hour reaction with HL197, showing far more reactivity than any other 5' oligo. The selective ligation of HL197 to the RNA provided confirmation that the cleavage occurred between the 11th and 12th bases of the native Tf1 mRNA.

4. Testing the role of RNase H activity in the cleavage of Tf1 mRNA.

Speculation about the source of the nuclease activity that catalyzed the cleavage between the 11th and 12th bases of the Tf1 mRNA led to the hypothesis that the RNase H domain of Tf1 RT was responsible. One argument against this possibility is that RNase H activity is thought only to cleave RNA when annealed to DNA and in the case of Tf1, the cleavage likely occurred in RNA that was annealed to RNA. Nevertheless, recent *in vitro* results show that RNase H from HIV-1 RT can cleave RNA in an RNA:RNA duplex (Götte, Fackler et al. 1995). Evidence that suggested Tf1 RNase H is required for RNA cleavage was provided by the result that the primer extension of RNA from Tf1 elements with mutations in the conserved sites of RNase H did not reveal cleaved RNA, while mutations in the YXDD motif did. The mutations in RNase H might have blocked cleavage altogether.

To ask whether Tf1 RNase H participates in the cleavage of the first 11 bases, the level of cleaved RNA produced by Tf1 with the D564N mutation was compared to levels made by Tf1 elements that carried mutations in RNase H active site residues and the D564N change. The mutations D710N and D779N in the RNase H domain of RT were chosen because our results indicated that these two residues were essential for RNase H function. Primer extension results showed that all of the strains with mutations in RNase H lost the ability to accumulate cleaved RNA. Although no RT antibodies are available to test the effect of the RNase H mutations on the levels of RT protein, the results of *in vitro* reverse transcriptase assays with artificial primer and template indicated that the D710N and the D779N mutations resulted in Tf1 VLPs that had wild-type levels of RT. These results showed that conservative mutations in the essential amino acid residues of Tf1 RNase H blocked cleavage, indicating that RNase H itself is required for cleavage activity.

The possibility that RNase H is responsible for cleaving the Tf1 transcript within the RNA duplex is intriguing because the *in vivo* activities of all RNase H enzymes are thought to be specific for RNA substrate that is annealed to DNA. Recent reports have indicated that in *in vitro* reactions, retroviral RNases H can degrade double-stranded RNA if Mn^{2+} is present (Blain and Goff 1993; Hostomsky, Hughes et al. 1994). The *in vivo* relevance of this activity is obscure and therefore it has been termed RNase H* by analogy with the convention for restriction enzymes with relaxed specificity (Hostomsky, Hughes et al. 1994). Detailed examinations of the *in vitro* activities of HIV-1 RNase H revealed that double-stranded RNA can be degraded even without the addition of Mn^{2+} if the enzyme is artificially arrested during polymerization (Götte, Fackler et al. 1995). This activity was shown to be 30-fold slower than the cleavage of RNA in RNA:DNA duplexes. The implication from these experiments is that RNases H may have the ability to cleave double-stranded RNA, but only if its active site is tightly bound to the substrate to allow sufficient time for the slow cleavage of duplex RNA. The cleavage of the Tf1 duplex RNA by Tf1 RNase H may require the enzyme to be specifically tethered to the cleavage site to allow a slow cleavage reaction to occur. If Tf1 RNase H does cut double-stranded RNA during priming, it would be a novel case of a physiological role for RNase H activity that cleaves RNA duplexes. An alternative explanation for the ability of Tf1 RNase H to cleave a specific nucleotide within an RNA structure is that the RNA itself may possess specific structural features that mimic RNA:DNA duplexes.

II. Self-primed reverse transcription of Tf1 requires the formation of an extensive RNA structure required for the cleavage reaction that generates the primer.

1. Identification of the putative RNA structure in the 5' untranslated region of the Tf1 mRNA.

Sequence analysis of the 5' untranslated region (5' UTR) in the Tf1 mRNA revealed a putative RNA structure of 89 nucleotides that encompasses the PBS. The proposed RNA structure is presented in Figure 3. This structure includes a hairpin and three helices formed by long range nucleotide interactions. In addition to the PBS helix described above, we identified a sequences that could potentially form a 5-bp helix upstream and a 11-bp helix downstream of the PBS helix. The 5-bp helix and the PBS helix are separated by a bulge of 4 nucleotides in the top strand. The location of the PBS in the center of this complex RNA structure led us to speculate that this region may be involved in the self-priming mechanism.

2. The mutagenesis of nucleotides within the RNA structure and the effects on Tf1 transposition.

To examine the function of the large RNA structure in transposition, we randomly mutagenized the region of secondary structure by PCR and isolated a total of 78 mutations. 34 of these represented single base mutations. Each of these reduced transposition significantly without affecting Tf1 protein levels. The mutant strains were subjected to the quantitative transposition assay and the results indicated that approximately half exhibited at least 50-fold less transposition activity while the bulk of the others showed 5 to 50-fold lower transposition. Figure 3 shows the sequence subjected to mutagenesis and the distribution of the single point mutations identified. All mutations were found to cluster within three duplex regions of the proposed RNA structure. 16 different mutations were found within the primer binding site duplex, 14 within the 11 base pair duplex, and 4 were identified in the 5 base pair duplex. The magnitude of the defects caused by each of the base changes is indicated in Figure 3 by the size of the bases. Each of the three regions that contained clusters of mutations possessed base changes that resulted in greater than 50-fold less transposition, indicating that all three regions make equally important contributions to transposition.

To test additional aspects of this putative structure, 8 site directed mutations were generated and each one is indicated in Figure 3 by an asterisk. Three mutations were made in the bottom strand of the 11 base pair duplex and one mutation was made in the top strand of the 5 base pair duplex because these regions were not subjected to the random mutagenesis. Although the mutations in the bottom strand of the PBS duplex exhibited modest effects on transposition, the G to U change in the top strand of the 5 base pair duplex reduced transposition by 116-fold. Two additional base changes made in the bulge between the 5 base pair region and the PBS duplex reduced transposition significantly. We also changed the 11th base of the transcript to an A to determine the importance of the sequence at the cleavage site. The T11A change resulted in a 50-fold drop in the frequency of transposition.

Although the sequence comprising the 12 base pair hairpin proposed in Figure 3 was subjected to random mutagenesis, only one mutation was identified that reduced transposition, and this change resulted in a significant drop in Tf1 protein levels. We tested directly the importance of the hairpin in the transposition mechanism by deleting its entire sequence. No loss in transposition activity was observed, suggesting that, at least under the conditions of our assay, the hairpin is not required for transposition.

3. Detection of nucleotide interactions by the examination of compensatory mutations.

We evaluated the duplex nature of the various regions within the proposed RNA structure by analyzing different sets of two mutations that, in combination, restore the potential to form the duplex interaction. We previously reported that compensating mutations in the PBS duplex resulted in rescue of transposition activity to within 85% of wild-type levels (Levin 1995). Four different sets of two mutations in other duplex regions were tested. The combination of two mutations located in the 11 base region or in the 5 base pair region exhibited rescue of transposition indicating that the two regions do form duplex structures *in vivo*. One set of two mutations resulted in such a significant level of rescue that the transposition frequency was 70% higher than produced by wild-type Tf1.

4. The mutations in the RNA structure result in reduced levels of reverse transcription.

We have developed a DNA blot method for the detection of Tf1 cDNA that enables us to measure amounts of mature double-stranded reverse transcript. *S. pombe* cell extracts are phenol extracted and ethanol precipitated. The resulting nucleic acid is restriction digested with enzymes that produce cDNA fragments with very different sizes from the plasmid Tf1 sequences. This method was used to screen each of the different

Tf1 elements with mutations in the RNA leader structure. The results showed that each of the mutants that caused reductions in transposition frequencies produced lower levels of cDNA. When the amounts of cDNA were quantitated, the reduction in reverse transcription caused by each mutation correlated well with the magnitude of their transposition defect. In addition, all four pairs of compensating mutations resulted in increased levels of reverse transcripts compared to that produced by the individual mutations. The correlation between the levels of transposition exhibited by the mutated copies of Tf1 and the quantity of cDNA produced by these elements indicated that the RNA structure was required for reverse transcription.

5. Mutations in the complex RNA structure resulted in reduced levels of minus-strand strong-stop DNA.

To examine the possibility that the mutations in the Tf1 RNA structure resulted in reduced levels of cDNA because of a defect in the self-priming mechanism, we measured the amounts of minus-strand strong-stop DNA produced by a representative set of 5 mutant Tf1 elements. *S. pombe* cells that were induced for the expression of the mutant versions of Tf1 were lysed and the extracts were fractionated on sucrose gradients to isolate the virus like particles (VLPs). DNA blot analysis of Tf1 VLPs from all of the 5 mutations showed reduced levels of strong stop DNA, suggesting that the defects in reverse transcription caused by these mutations affected the priming of cDNA synthesis.

6. Defects in the RNA structure result in low levels of cleaved Tf1 mRNA.

To determine whether the mutations in the RNA structure block reverse transcription by causing a defect in the generation of the Tf1 primer, we adapted the primer cleavage assay for use with the RNA structure mutants. Fourteen of the Tf1 mutations that caused defective transposition were subcloned into a Tf1 plasmid that also contained a mutation in the DNA polymerase domain of RT. As described above, the RT mutation results in the accumulation of cleaved Tf1 mRNA and therefore allowed us to measure the level of cleaved Tf1 mRNA in the cells that contained the mutated RNA structures. Primer extension analysis of RNA extracted from these strains was then used to quantify the amount of cleaved Tf1 mRNA. The results showed that 13 of the 14 mutations in the RNA structure resulted in significantly lower levels of cleaved Tf1 mRNA, indicating that the defects in reverse transcription caused by these mutations resulted from a failure to prime minus-strand strong-stop DNA synthesis. Surprisingly, the T11A mutation exhibited wild-type levels of cleaved Tf1 mRNA, indicating that the lack of minus-strand strong-stop DNA caused by this base change resulted not from a lack of cleaved primer but from an inability of RT to extend the primer. The primer generated by Tf1 with the T11A mutation was predicted not to be extended by RT because the mutant primer possessed a single base mismatch at its 3' terminus. The fulfillment of this prediction provided substantial support for the model that the first 11 bases of the Tf1 transcript serves as the primer for minus-strand reverse transcription.

7. Tf1 defines a family of eight retrotransposons that appear to undergo self-primed reverse transcription.

We previously reported that besides Tf1, two other retrotransposons, Tf2 and CfT-1 have transcripts that lack candidates for tRNA primers and also have complementarity between their primer binding sites and sequence further upstream in the same mRNA. Although Tf2 was isolated from *S. pombe* and is closely related to Tf1, CfT-1 was isolated from the fungal pathogen, *Cladosporium fulvum*. These observations led us to propose that these three elements define a family that undergo self-primed reverse transcription.

Several other LTR-containing retrotransposons from fungal sources have been entered into DNA databases. After examining these sequences we find that there are 5 new elements from different pathogenic fungi that appear to be members of the self-priming family. Afut1 is a retrotransposon recently isolated from *Aspergillus fumigatus* (Neuveglise, Sarfati et al. 1996). Boty is a retrotransposon isolated from the pathogenic fungus *Botrytis cinerea* (Diolez, Marches et al. 1995). The fungus that causes rice blast, *Magnaporthe grisea*, contains two retrotransposons, Grasshopper (Dobinson, Harris et al. 1993) and Maggy (unpublished) . Skippy is a retrotransposon that is 8.5 kb in length and was isolated from *Fusarium oxysporum* (Anaya and Roncero 1995). All five of these transposons are LTR-containing elements that lack tRNA candidates for primers and also have complementarity between their primer binding sites and sequence further upstream. In addition, sequence analysis of the RT coding sequences indicates that all eight of the self-priming transposons are more closely related to each other than to other retrotransposons. We are continuing our analysis of the sequences in hopes of identifying regions of extended RNA structure similar to the Tf1 structure.

III. The contribution of Tf1 proteins to integration.

1. A random mutagenesis of Tf1 and the identification of mutations that caused reduced transposition.

We described in our last annual report our approach to identify Tf1 proteins that are critical for transposition. We chemically mutagenized a preparation of plasmid DNA that contained a wild-type version of Tf1. Once this DNA was transformed into yeast, approximately 100 strains were identified that exhibited defects in transposition. To focus on mutations that blocked the integration step of transposition, the defective strains were further characterized using a homologous recombination assay that allowed us to identify a subclass of strains that produced normal levels of reverse transcript. DNA blot analysis was used to confirm that 9 strains with versions of Tf1 defective for transposition were nevertheless capable of producing high levels of reverse transcript. To understand how these mutations inhibited the integration activity of Tf1, we identified which restriction fragments of the defective plasmids contained base changes. Our previous report indicated that the bulk of the mutations were positioned in the coding sequence of IN as was expected based on the lack of integration exhibited by these strains.

2. Mutations in RNase H that block transposition but not reverse transcription.

In the previous report we described the surprising observation that two of the strains defective for transposition carried Tf1 mutations that were not located in IN but instead were found to be in the RNase H domain of RT. The identification of mutations in RNase H that inhibited integration and not reverse transcription was unexpected because the RNase H domain of other reverse transcriptases is known to be required for normal reverse transcription. Retroelements that lack RNase H activity cannot undergo full reverse transcription because minus strand transfer is inhibited (Blain and Goff 1995). Because the DNA blot analysis of the two strains with mutations in RNase H showed that high levels of mature reverse transcript were produced, we speculated that the defects in transposition were due to the loss of specialized functions that RNases H possesses such as the removal of the minus strand primer or the generation of the plus strand primer (Champoux 1993). Either of these defects could potentially inhibit IN from joining the 3' ends of the cDNA to the 5' ends of the target. In support of this model, we found that one of the RNase H mutations, S749L was 3 amino acid upstream of the critical active site glutamic acid, a position that could contribute the specificity required for the specialized functions of RNase H without being required for catalytic activity. In addition, we found that most retroviral RNases H possess either a serine or a threonine 4 or 5 residues upstream of the conserved glutamic acid and a recent crystal structure of the HIV RNase H bound to DNA showed that this residue forms a hydrogen bond with a template nucleotide (unpublished, E. Arnold). For these reasons, we decided to examine the possibility that the RNase H mutations in Tf1 affected the structure of the 5' ends of the cDNA. We employed primer extension assays to identify the exact termini of the DNA sequence and to measure the levels of residual RNA primer attached to these ends. The results indicated that there were no observable differences between the 5' ends of the reverse transcripts produced in log-phase growth by wild-type Tf1 compared to that produced by a version of Tf1 with the S749L mutation. Thus, it is unlikely that the transposition defect caused by the S749L mutation was due to the lack of the specialized activities of RNase H that process the 5' ends of the reverse transcript.

Alternative explanations for the defect in integration caused by the RNase H mutations include the possibility that RNase H contributes directly to the integration mechanism and a critical interaction with IN was disrupted by the mutations. Another possibility is that the RNase H mutations resulted in a reduction in the activity of the enzyme that was manifested by a reduction in the rate that reverse transcription was completed. Since the bulk of reverse transcription does not occur until cells reach late log-phase, a delay in cDNA synthesis could result in lower levels of reverse transcript during a period in cell growth when integration could be more efficient. The DNA blot analysis might not have revealed a simple reduction in the rate of cDNA synthesis because these cultures were harvested in stationary phase. Quantitative measurements of the amounts of cDNA is cells throughout liquid culture growth indicated that the most characterized RNase H mutation had no effect on the rate of reverse transcription.

3. The sequence analysis of the IN mutations.

As the result of recent sequencing efforts, we have found that 5 of the mutant strains isolated from the random mutagenesis of Tf1 carried base changes in IN. As indicated above, mutations in IN were expected because all 9 strains defective for transposition were still able to produce high levels of reverse transcript. Two of these mutations fell in the N-terminal domain which contains a highly conserved Zn binding motif. Although this domain contains about 130 amino acids based on alignments with other integrases, the two mutations L929I and P903L were found to be within 26 residues of each other and were located on either side of two conserved histidines that are known to bind Zn in other IN proteins. This observation suggested that these histidines are critical residues in the Tf1 IN and are likely required because of their interaction with Zn. Another mutation, S1010L was located in the catalytic core domain 23 amino acids downstream of an aspartic acid residue that is conserved among all integrases. A fourth mutation, G1311T, was located in the DNA binding domain located in the C-terminus of the Tf1 integrase suggesting that the C-terminal domain in Tf1 IN is also critical. The fifth mutation in IN was E1142K and was found to be between the catalytic core domain and the C-terminal domain. Conclusions based on the position of this mutation were more difficult because it was found to be one of two mutations within the same Tf1 plasmid. The other base change was interesting because it fell within the RNase H domain of RT as did two other single mutations discussed earlier. To determine whether the E1142K mutation has a phenotype by itself, or whether the mutation is RNase H caused the defect in transposition, we are recreating these changes individually.

IV. The characterization of host mutations that inhibit transposition.

1. The isolation and initial characterization of host mutations that reduce transposition.

In order to improve our understanding of the retrotransposition process and by analogy, retrovirus infection, we initiated a large scale genetic screen for *S. pombe* strains that were defective for transposition. As reported last year, cultures of *S. pombe* that contained our Tf1 assay plasmid were mutagenized with nitrosoguanadine under conditions that caused 80% lethality on minimal medium. After these strains were colony purified, 5,000 were patched onto our transposition assay media and screened for reduced frequencies of transposition. Approximately 176 strains from two mating types reproducibly transposed at significantly lower levels than the parent strains. All these candidates were tested for the presence of single genetic lesions affecting transposition and for the magnitude of the deficiency caused by each mutation. As a result, we now focus our experiments on 3 mutant strains that had transposition frequencies 10-fold lower than that of wild type cells. Complementation analysis demonstrated that each of the strains contained mutations in different genes we named *Hop2, hop3* and *Hop5.*

Further analysis of the defective strains was designed to identify which transposon processes were inhibited by each of the mutations. Immunoblot analysis revealed that the mutations in Hop2 greatly reduced expression of the Tf1 proteins. RNA analysis indicated that the low levels of Tf1 proteins produced in this strain was not due to reduced levels of mRNA. Consistent with the lack of Tf1 proteins was the observation that the strain containing the Hop2 mutation produced no detectable cDNA as determined by DNA blot analysis. In contract to this strain, the cells with mutations in the Hop3 and Hop5 genes contained wild-type levels of cDNA indicating that the defects in these cells inhibited transposition after reverse transcription. To address whether the reverse transcript produced in these cells was transported into the nucleus, we measured the frequency of homologous recombination that occurred between the cDNA and the plasmid copies of Tf1. The low levels of recombination observed in the strains with the Hop3 and Hop5 mutations suggested that the Tf1 cDNA was not transported to the nucleus.

2. Recent characterization of strains with the Hop3 mutation.

Earlier studies of cells that contained the Hop3 mutation indicated that the levels of Tf1 proteins were normal in cells during log-phase growth but were significantly reduced in cells that reached stationary-phase. To test whether this effect was the result of the Hop3 mutation, we crossed our mutant strain with wild-type cells and isolated progeny that carried the Hop3 defect. After three rounds of backcrosses we tested the resulting strains to measure the expression of Tf1 proteins in cells that had reached stationary phase. We were surprised to find that each of the four spores dissected from tetrads developed into strains that in stationary phase contained wild-type levels of Tf1 protein even though two of the four strains derived from each tetrad possessed the Hop3 mutation. We concluded that the mutation in the Hop3 gene caused no effect on the levels of Tf1 proteins.

We have previously tested the Hop minus strains for the ability to produce reverse transcripts that can undergo homologous recombination with plasmid sequences. These experiments were conducted to identify cDNA in the nucleus. Both the Hop3 minus and

Hop5 minus strains were defective for this recombination suggesting that the mutations resulted in less transport of cDNA to the nucleus. Another interpretation was that the host factors required for general homologous recombination also play a role in transposition. To explore this possibility we have used several types of assays to test the recombination properties of strains containing the Hop3 mutation. We first tested cells with the Hop3 defect for sensitivity to ultraviolet light. Extensive analysis revealed that cells in liquid cultures grown to stationary phase showed no added sensitivity to UV due to the Hop3 mutation. Careful examination of cultures in log-phase growth revealed that the Hop3 mutation caused a 2-fold increase in sensitivity to UV. The small difference in resistance to UV due to the Hop3 mutation made it difficult to associate with a significant effect on recombination. We are in the process of evaluating the effect of the Hop3 mutation on integrative recombination of a plasmid lacking Tf1 with a homologous site in the genome. In addition, we are testing the effect of the Hop3 mutation on homologous recombination between two allelic sequences in diploid cells.

Because all of our characterization of strains carrying the Hop3 mutation were limited to one allele, several efforts have been made to isolate the Hop3 gene in order to generate a strain with a Hop3 deletion that could be tested for transposition and recombination phenotypes. Two separate genomic libraries that contained 10 kb inserts were obtained from other labs and amplified here. Although over 80,000 transformants of a strain with the Hop3 mutation were screened for increased transposition, no candidates were obtained. In addition we are screening 50,000 transformants of a cDNA library and have not identified any candidates that fully complement the Hop3 defect. We concluded that either these libraries were incomplete or that multicopy plasmid expression of the Hop3 gene was either lethal or unable to complement the genomic defect. Several approaches are underway to clone the Hop3 gene that address these possibilities. The creation of 4 new libraries is described later in this report.

Significance to Biomedical Research and the Program of the Institute.

The medical significance of our research stems from the close relationship between retroviruses and retrotransposons. The high level of similarity in structure between these two types of retroelements results in the large number of mechanisms that the elements share. The mechanism of IN mediated insertion for retrotransposons and retroviruses has been shown to require the same reaction intermediates (Eichinger and Boeke 1988). The early steps of reverse transcription are primed from the same position of the elements and both types of retroelements use polypurine sequences for priming plus strand reverse transcription. The structure of transposon virus-like particles is analogous to retrovirus particles in their composition of capsid and Pol proteins. The medical importance of retroviruses is dominated by the role of HIV in the AIDS epidemic. HIV is a retrovirus that appears to undergo the same types of reverse transcription, proteolysis, integration and particle formation as do retrotransposons. Because many aspects of retrovirus function, including particle assembly, reverse transcription, nuclear entry and chromosome integration, are difficult to study in hosts that possess a high level of genetic complexity, the molecular genetic analysis of retrotransposons in yeast provides a promising approach to answering many of the important questions that are at the center of the AIDS crisis.

The study of transposons in general and retrotransposons in particular is relevant to the understanding of neoplastic disease mechanisms. Transposons are endogenous mutagens of cells; chemical mutagenesis is strongly correlated with carcinogenesis; therefore studies of these endogenous biological mutagens is highly pertinent to an understanding of cancer. Moreover, the growth of a wide variety of tumors is associated with activation of endogenous retroviral expression and rearrangements of the genomic DNA in tumor cells. Many studies clearly indicate that the activation of cellular oncogenes is directly responsible for mammalian neoplasms. In many cases, such gene activation is caused by retrovirus or retrotransposon insertion into a proto-oncogene locus. For this reason, the thorough understanding to be gained from studies of retrotransposons inhabiting the genomes of genetically tractable organisms, particularly yeast, is directly relevant to the understanding of both oncogene activation mechanisms and the DNA rearrangements that occur in cancer cells.

Proposed Course

I. The identification of Tf1 virus-like particles as intermediates of transposition.

Despite the success of our genetic experiments that show Tf1 is an active LTR-containing retrotransposon that encodes a functional RT and IN, we have little evidence that Tf1 transposition proceeds by a process that includes virus-like particles as essential intermediates. This issue is especially important for the characterization of Tf1 transposition because the translation of equal amounts of Gag and Pol protein is particularly unusual and could result in a transposition mechanism that may not require the formation of virus-like particles. By studying the behavior of Tf1 protein in sucrose gradients, we found the Gag protein sedimented with a velocity typical of transposon particles. In addition, the IN and minus-strand strong-stop DNA both sedimented to the same position in the sucrose gradient as did the Gag protein, suggesting that the Gag, IN and DNA were associated within the same particle structure (Levin, Weaver et al. 1993). Direct evidence for a interaction between the Tf1 proteins and cDNA would be valuable in light of the difficulty we have had observing Tf1 particles by electron microscopy. Although we attribute this problem to the low levels of Tf1 protein produced by the *nmt1* promoter, the possibility exists that Tf1 does not form the type of virus-like particles that Ty1 and Ty3 produce.

1. The function of Gag in transposition.

We have shown that in stationary-phase cells, Tf1 Gag is present in 26-fold excess compared to IN and that the molecular weight of Gag is 27 kDa, a size typical of other Gag proteins. However, we have no genetic data indicating that Tf1 Gag is essential for transposition. We will PCR-amplify sections of Gag to create a set of six 10-amino-acid deletions evenly spaced across the coding sequence of Gag. We will also generate a complete deletion of Gag sequences. The Gag deletion plasmids will be transformed into our *S. pombe* strains and tested to determine what effect the deletions have on the Tf1 transposition frequency. Protein extracts from cells containing each Gag deletion will be loaded onto sucrose gradients and the fractions will be immunoblotted to correlate any defects in transposition with changes in sedimentation behavior. If IN is truly associated with Gag in particle structures largely composed of Gag, the absence of Gag should drastically reduce the sedimentation velocity of IN in the sucrose gradients.

2. Identification of direct associations between Tf1 Gag, RT, and IN in virus-like particles.

Although Tf1 Gag, RT, and IN have been found to cosediment in sucrose gradients, suggesting they coassemble into particles, the possibility exists that they individually assemble into separate particles composed of one type of protein each. This question is very important in the case of Tf1 protein isolated from log-phase cells since the equal molar amounts of Gag and IN may simply be forming separate aggregates that are unassociated and coincidentally cosediment in sucrose gradients. We will choose 6 sites in Gag to insert the FLAG epitope (Kodak) sequence of 8 amino acids. This 8 amino acid sequence binds with high specificity to a monoclonal antibody affinity matrix and will be used to purify Tf1 particles containing FLAG-tagged protein from *S. pombe* extracts. The *nmt1*-Tf1 clones containing the FLAG epitope will be integrated into the *S. pombe* genome to allow for low expression of tagged protein and coassembly of tagged protein with wild-type Tf1 protein expressed from our high copy *nmt1*-Tf1-*neo* assay plasmid. These cells will be tested for transposition to evaluate the effect of FLAG epitope on the function of the hybrid particles. The strains showing wild-type transposition will be extracted from stationary-phase cultures to identify which tagged positions allow for the best purification of protein by the affinity gel. The affinity-purified fractions containing tagged protein will be immunoblotted to determine whether associations between Gag, RT, and IN can be detected. The tagged material will also be tested for the copurification of reverse transcripts and Tf1 mRNA as would be expected if they are packaged in typical virus-like particles. The tagged Gag protein will be purified from log-phase cells to reveal whether the high levels of IN in these cells are truly associated with Gag in an unusual form of particle.

3. Detection of Tf1 virus-like particles by electron microscopy.

The visualization of Tf1 virus-like particles by electron microscopy would help to answer several questions about the role in transposition of the Gag and IN complexes that sediment rapidly in sucrose gradients. Although we routinely observe that Gag, IN, RT and cDNA cosediment as large complexes in sucrose gradients, we have never been able to visualize virus-like particles by the electron microscopic examination of sucrose gradient fractions or of whole-cell thin sections. One possibility is that Tf1 transposition does not occur via typical virus-like particles and the material that rapidly sediments in sucrose gradients is just nonspecific aggregates of Tf1 protein. A likely explanation for our inability to visualize particles may be that the amounts of transposon proteins produced by our assay plasmid is at least 10-fold lower than what is expressed by the GAL promoter of the Ty1 plasmids of *S. cerevisiae,* and therefore the numbers of Tf1 particles in the expression strains may be low and not visually different from that seen in the nonexpression control strains. To avoid this possibility, we will use the electron microscope to examine the purified Tf1 material isolated by the tagging techniques described in the above section. The Tf1 protein will be imbedded and sectioned for visualization by transmission microscopy. We will also attempt to visualize particles by negative staining techniques. All of the electron microscopy will be performed by a service facility available to NIH labs.

II The identification of sequences in Tf1 required for the packaging of mRNA into virus-like particles (VLPs).

Although the particles of retroviruses are readily obtained from culture medium, the isolation of retrotransposon particles has been much more difficult because they must be purified from cytoplasmic fractions. Therefore, it has been challenging to identify conditions under which packaged transcripts can be purified from the bulk of cellular mRNA. Despite the demonstration by us and others that retrotransposon mRNAs do cofractionate with VLPs, we are unaware of any experiment showing that the packaged mRNA can be purified away from the cellular mRNA and thus demonstrate that the RNA in the particle is in a separate pool from bulk RNA. One reason we are interested in establishing these conditions is because some of the point mutations in Tf1 that we introduced into the 5' UTR may inhibit packaging. The possibility exists that some of the mutations that resulted in less cleavage of the mRNA did so in an indirect way that was due to the exclusion of the mRNA from particles. The complex RNA structure in the 5' UTR may possess duel functions. The PBS duplex may function to initiate priming while the 11 base duplex may contribute to packaging. An additional motivation for developing a packaging assay is to identify other regions of the Tf1 mRNA required for packaging. This would allow us to compare the packaging mechanisms of Tf1 to those of retroviruses.

The protocol we use to isolate Tf1 VLPs results in fractions that contain high levels of cellular mRNA. We will modify the parameters of the sucrose gradient fractionation to separate the rapidly sedimenting VLPs from the bulk of the cellular mRNA by significantly shortening the centrifugation time. We now find that Tf1 particles sediment to the bottom of a 20%-70% sucrose gradient within 20 minutes. In addition, we are developing methods to epitope tag Gag (described above) that will allow us to greatly improve the purity of our VLP preparations. We will analyze these fractions for Tf1 mRNA as well as several unrelated cellular transcripts to gauge the success of this method of purification.

Once an assay for mRNA packaging is established, we will select a representative number of mutations in each region of the RNA structure to be tested for defects in packaging. In this way we will be able to determine which sequences are necessary for packaging. The assay will also be used to identify which Tf1 sequences are sufficient for packaging. A two plasmid system will be created in which the Tf1 proteins will be supplied by a helper version of Tf1 that lacks the genetic marker for transposition. The second plasmid will carry a mini copy of Tf1 that contains the neo-marker for transposition. We will make a series of large deletions in the mini Tf1 to determine which regions of the transposon can be removed without reducing the packaging efficiency of the neo-tagged RNA. The effect of each deletion on packaging can be monitored with the transposition assay.

III. Self-priming of reverse transcription

1. The functional analysis of sequences adjacent to the complex RNA structure in the 5' UTR of the Tf1 mRNA.

The sequence of the large RNA structure previously found to be required for cleavage of the Tf1 mRNA comprises about half of the 5' UTR. Other sequences in the 5' UTR possess the potential to form RNA structures and may therefore participate in the transposition mechanism. We will generate a set of Tf1 plasmids that contain mutations across the regions of potential secondary structure using fusion PCR to substitute 20 consecutive nucleotides. Each of these plasmids will be transformed into *S. pombe* and tested for transposition activity. The strains that exhibit reduced transposition will be examined for defects in protein expression, reverse transcription. If our efforts to develop an assay for the packaging of RNA in Tf1 VLPs are successful, we will also measure the effect of the leader mutations on RNA packaging. Sequences that are identified as critical for transposition and that exist in regions of potential RNA structure will be tested for their ability to form structures *in vivo*. Mutations will be introduced into the opposite strand of the potential structures to reestablish complementarity. If the defects in transposition can be rescued by this approach, we will conclude that the potential structure in question does form.

2. The analysis of Tf1 VLPs for the ribonuclease activity that cleaves the Tf1 mRNA to generate the primer for reverse transcription.

The mutagenesis data indicating that Tf1 RNase H is required for cleavage of the primer presented us with important unanswered questions. Although mutations in RNase H resulted in inhibition of the cleavage reaction, there was no direct evidence that the RNA was cleaved by the Tf1 RNase H. We have made several attempts to detect cleavage activity in the sucrose fractions that contain Tf1 VLPs. When the Tf1 mRNA leader is generated *in vitro* and added to the sucrose fractions, no specific cleavage was detected. Instead, we observed random degradation that was presumably due to contaminating RNases. When highly purified preparations of epitope tagged VLPs become available, we will test them for the ability to cleave Tf1 mRNA specifically between the 11th and 12th bases. If such an activity can be observed, particles containing defective RNase H will also be tested. To determine whether RNase H is the only particle component required for cleavage, we will purify epitope tagged RNase H from *S. pombe* extracts.

3. Retroviral RNase H recognition of the self- priming RNA structure.

One reason to consider the role of other nucleases in the cleavage of the Tf1 mRNA is that the RNA structure presumably contains no RNA:DNA duplexes and therefore is not a typical RNase H substrate. Unfortunately, we have been unable to obtain purified Tf1 RT in a soluble form that could be used to test if the enzyme does possess the ability to cleave the Tf1 RNA. We will continue our efforts to purify Tf1 RT from various expression systems using tagged epitopes. We will also attempt to renature the insoluble RT that we have previously isolated. An additional method we will use to address the direct role of RNase H in the cleavage reaction will be to test purified RT enzymes from MuLV and HIV in reactions that contain *in vitro* transcribed Tf1 mRNA. Should either of these enzymes cleave the RNA, we will determine if mutations in RNase H active site residues inhibit cleavage. If so we will have learned that RNase H enzymes do possess the catalytic activity necessary for the reaction. In addition, we would conclude that the cleavage of the Tf1 mRNA *in vivo* is not the result of a specialized property of the Tf1 RNase H but is instead a unique feature of the RNA structure that in some way mimics the properties of an RNA:DNA duplex

IV. The role of RNase H in the integration mechanism.

1. Tf1 RNase H will be examined for a general role in integration.

We have previously described the identification of mutations in Tf1 that inhibited transposition but had minimal effect on the levels of reverse transcription. The strains carrying these mutations were identified as colonies that underwent high levels of homologous recombination between cDNA and plasmid sequences but exhibited low levels of transposition activity. DNA blot analysis confirmed that these strains produced high levels of full-length double-stranded reverse transcript. Although most of these mutations in Tf1 mapped in the IN protein, we were surprised to find that two were in RNase H. This was an unexpected result because previous studies of RNase H mutants indicated that the synthesis of mature double-stranded cDNA required RNase H activity to release single stranded DNA intermediates from their RNA templates (Champoux 1993; Blain and Goff 1995). In addition, we have made amino acid substitutions in critical residues of Tf1 RNase H and found that RNase H activity is absolutely required for transposition and cDNA synthesis (Levin 1996).

One explanation for these unusual mutants is that they specifically affect the ability of RNase H to cleave the residual RNA primers off the 5' ends of the cDNA without reducing the enzyme's general ability to degrade RNA annealed to DNA. The result of this defect would be that RT could produce completed cDNA but the presence of the RNA primers would inhibit the integration reaction. We have tested this hypothesis by using a primer extension method and have found no evidence for defects in the processing of the RNA primers.

We will test whether RNase H can interact directly with IN during integration using the two-hybrid system of *S. cerevisiae* . The three separate domains of IN, the Zn binding domain, the catalytic core, and the DNA binding domain, will be fused to the lexA DNA binding domain in two-hybrid test plasmids. These domains will be tested for interactions with the RNase H domain of the Tf1 RT. If strong interactions are detected, we will introduce the RNase H mutations that inhibit integration to determine whether these residues of RNase H contribute to interactions with IN.

To determine whether the other amino acids in RNase H adjacent to the integration defective mutation S749L also contribute to integration, we will generate single amino acid changes within a stretch of 12 residues that contain several highly conserved amino acids. These mutations will be produced randomly so that additional changes in S749 can be isolated. Once a large set of mutagenized plasmids are isolated, they will be transformed into *S. pombe* and characterized using the transposition and recombination assays.

If other mutations in RNase H can be identified that block integration we will expand our set of RNase H mutations to enlarge our map of amino acid residues that participate in this unusual function. We will specifically mutagenize Tf1 RNase H sequence and screen for elements that have reduced transposition activity but produce wild-type levels of cDNA as measured by our homologous recombination assay. The position of these mutations will be determined and their three dimensional locations modeled using the available structures of other RNase H proteins (Katayanagi, Miyagawa et al. 1990; Yang, Hendrickson et al. 1990; Davies, Hostomska et al. 1991; Kohlstaedt, Wang et al. 1992). By identifying the number and type of amino acids in RNase H that contribute to integration and not reverse transcription, we hope to learn more about why these mutations block integration.

2. The identification of mutations in Tf1 that suppress S749L.

One possible explanation for why the S749L mutation caused a defect in integration is that RNase H may interact directly with IN to "hand-off" the cDNA or to participate directly in the integration reaction. The fact that crystallographic studies of IN and RNase H showed that they possess very similar structure suggests that these two proteins could multimerize. To identify potential interactions between the mutated version of RNase H and IN, we will PCR mutagenize IN in a version of Tf1 that contains the S749L mutation. We will transform the mutagenized DNA into *S. pombe* and screen for transformants that transpose with elevated frequencies. The positions of the suppression mutations will be identified using a subcloning and sequencing. We will further our analysis of potential interactions with RNase H by testing the newly isolated suppressor mutations to determine is they exhibit allele specificity with other mutations in RNase H.

V. The identification of host genes required for transposition.

1. Host functions required for late steps in the transposition process.

As the result of a large-scale screen of mutagenized strains for defects in Tf1 transposition, we have identified two genes, hop3 and hop5 that contribute significantly to late steps of the transposition process. One recessive mutation was isolated in each of these genes and both mutations result in a 8-fold reduction in transposition. The analysis of strains containing the hop3-1 and hop5-1 mutations indicated that normal levels of Tf1 proteins and cDNA were produced. These results suggested that the defects in transposition inhibit activities required after reverse transcription takes place. The immediate goal of the experiments in this section is to identify the functions in the transposition process of the products encoded by the *hop3* and *hop5* genes. Our initial means of achieving this goal will be to isolate the genes by transforming a genomic library into the mutant strains and screening candidates for the complementation of the hop$^-$ phenotype of the transposition assay. Once clones of these hop genes are isolated and sequenced, we will use this information to determine the full role of each gene in transposition by generating *S. pombe* strains that contain complete deletions of the hop genes.

Our previous efforts to isolate *hop* genes have failed either because the two genomic libraries we received were incomplete or because the hop genes as expressed in multicopy vectors are unable to complement the genomic mutations. To address these problems we are generating our own *S. pombe* genomic library taking steps to make certain that it will be complete. In case the expression of the *hop* genes in multicopy is lethal or is unable to complement the genomic mutations, we will develop a single-copy integration library and introduce it into the genome of the mutant strains. This bank of integrated strains will be screened for isolates that have recovered wild-type levels of transposition activity. Genetic tags included in the DNA to be integrated will allow for the efficient isolation of the hop genes. Our design of this integrating library parallels that initially developed for use with *Saccharomyces cerevisiae* (Burns, Grimwade et al. 1994).

As an independent means of isolating the hop3 gene, we are screening an *S. pombe* cDNA library for plasmids that complement the defect in transposition. 50,000 transformants have been screened and as a result four plasmids were identified that partially rescue the defect in transposition. Two of these plasmids contain coding sequence from a gene that has strong similarity to amino-levulinic acid dehydrotase. This enzyme catalyzes the second step in the synthesis of heme, a prosthetic group required by many proteins necessary for respiration. We are currently testing whether this sequence is from the hop3 gene and whether the suppressing plasmid rescues the transposition defects caused by mutations in hop5 or hop2.

2. A host function required for an early step in Tf1 transposition.

A third host gene found to contribute to transposition was identified during the screen of mutagenized strains. A recessive mutation in hop2 results in a 8-fold reduction in transposition and a substantial reduction in cDNA synthesis. Immunoblot analysis of this strain revealed that the hop2-1 mutation caused a reduction in Tf1 protein accumulation that was not caused by reduced transcription of the nmt1 promoter. In an effort to identify the function of hop2, we are attempting to isolate the hop2 gene by transforming the mutant strain with the best available genomic libraries to identify genomic inserts that complement the transposition defect.

References

Anaya, N. and M. I. Roncero (1995). "Skippy, a retrotransposon from the fungal plant pathogen Fusarium oxysporum." Mol Gen Genet **249**(6): 637-47.

Atwood, A., J. Lin, et al. (1996). "The retrotransposon Tf1 assembles virus-like particles with excess Gag Relative to Integrase because of a Regulated Degradation Process." Mol. Cell. Biol. **16**: 338-346.

Blain, S. and S. Goff (1995). "Effects on DNA synthesis and translocation caused by mutations in the RNase H domain of Moloney Murine Leukemia virus reverse transcriptase." J. Virol **69**: 4440-4452.

Blain, S. W. and S. P. Goff (1993). "Nuclease activities of Moloney murine leukemia virus reverse transcriptase. Mutants with altered substrate specificities." J Biol Chem **268**(31): 23585-92.

Burns, N., B. Grimwade, et al. (1994). "Large-scale analysis of gene expression, protein localization, and gene disruption in Saccharomyces cerevisiae." Genes Dev **8**(9): 1087-105.

Champoux, J. (1993). Roles of ribonuclease H in reverse transcription. Reverse Transcriptase. A. Skulka and S. Goff. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press: 103-117.

Davies, J., Z. Hostomska, et al. (1991). "Crystal structure of the ribonuclease H domain of HIV-1 reverse transcriptase." Science **252**: 88-95.

Diolez, A., F. Marches, et al. (1995). "Boty, a long-terminal-repeat retroelement in the phytopathogenic fungus Botrytis cinerea." Appl Environ Microbiol **61**(1): 103-8.

Dobinson, K. F., R. E. Harris, et al. (1993). "Grasshopper, a long terminal repeat (LTR) retroelement in the phytopathogenic fungus Magnaporthe grisea." Mol Plant Microbe Interact **6**(1): 114-26.

Eichinger, D. J. and J. D. Boeke (1988). "The DNA intermediate in yeast Ty1 element transposition copurifies with virus-like particles: cell-free Ty1 transposition." Cell **54**(955): 955-966.

Götte, M., S. Fackler, et al. (1995). "HIV-1 reverse transcriptase-associated RNase H cleaves RNA/RNA in arrested complexes: implications for the mechanism by which RNase H discriminates between RNA/RNA and RNA/DNA." Embo J **14**(4): 833-41.

Hostomsky, Z., S. H. Hughes, et al. (1994). "Redesignation of the RNase D activity associated with retroviral reverse transcriptase as RNase H." J Virol **68**(3): 1970-1971.

Hsu, M., S. Eagle, et al. (1992). "Cell-free synthesis of the branched RNA-linked msDNA from retron-Ec67 of *Escherichia coli*." J. Biol. Chem. **267**: 13823-13829.

Katayanagi, K., M. Miyagawa, et al. (1990). "Three dimensional structure of ribonuclease H from E. coli." Nature **347**: 306-309.

Kohlstaedt, L., J. Wang, et al. (1992). "Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor." Science **256**: 1783-1790.

Lampson, B., J. Sun, et al. (1989). "Reverse Transcriptase in a Clinical Strain of *Escherichia coli*: Production of Branched RNA-Linked msDNA." Science **243**: 1033-1038.

Lampson, B. C., M. Inouye, et al. (1989). "Reverse transcriptase with concomitant ribonuclease H activity in the cell-free synthesis of branched RNA-linked msDNA of *Myxococcus xanthus*." Cell **56**: 701-707.

Levin, H. L. (1995). "A Novel mechanism of self-primed reverse transcription defines a new family of retroelements." Mol Cell Biol **15**: 3310-3317.

Levin, H. L. (1996). "An unusual mechanism of self-primed reverse transcription requires the RNase H domain of reverse transcriptase to cleave an RNA duplex." Mol Cell Biol **in press.**

Levin, H. L. and J. D. Boeke (1992). "Demonstration of retrotransposition of the Tf1 element in fission yeast." EMBO J. **11**: 1145-1153.

Levin, H. L., D. C. Weaver, et al. (1993). "Novel gene expression mechanism in a fission yeast retroelement: Tf1 proteins are derived from a single primary translation product." EMBO Journal **12**(12): 4885-4895.

Mizrahi, V., R. Brooksbank, et al. (1994). "Mutagenesis of the conserved aspartic acid 443, glutamic acid 478, and aspartic acid 498 residues in the ribonuclease H domain of p66/p51 human immunodeficiency virus type I reverse transcriptase." Journal of Biological Chemistry **269**: 19245-19249.

Moore, M. and P. Sharp (1992). "Site-Specific Modification of Pre-mRNA: The 2'-Hydroxyl Groups at the Splice Sites." Science **256**: 992-997.

Neuveglise, C., J. Sarfati, et al. (1996). "Afut1, a retrotransposon-like element from aspergillus fumigatus." Nucleic Acids Research **24**: 1428-1434.

Repaske, R., J. Hartley, et al. (1989). "Inhibition of RNase H activity and viral replication by single mutations in the 3' region of moloney murine leukemia virus reverse transcriptase." Journal of Virology: 1460-1464.

Yang, W., W. Hendrickson, et al. (1990). "Structure of ribonuclease H phased at 2 Å resolution by MAD snalysis of the selenomethionine protein." Science **249**: 1398-1405.

Model for self-priming of Tf1 reverse transcription

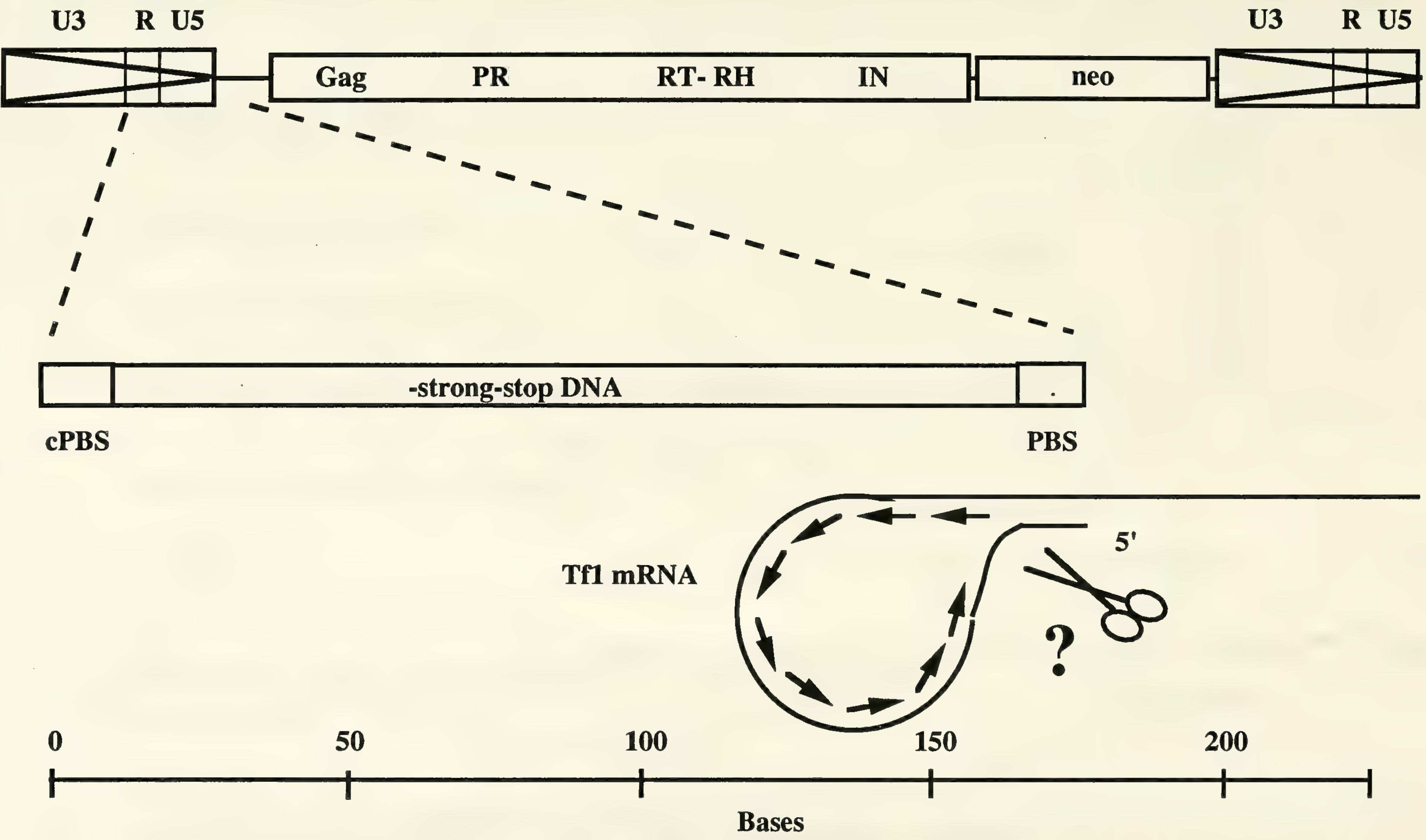


Figure 1. The model for self-priming of Tf1 reverse transcription. The first 11 bases of the Tf1 mRNA anneal to the PBS and a cleavage reaction generates the primer for reverse transcription.

Splint ligation of Tf1 mRNA to oligo for cleavage at the 11th base

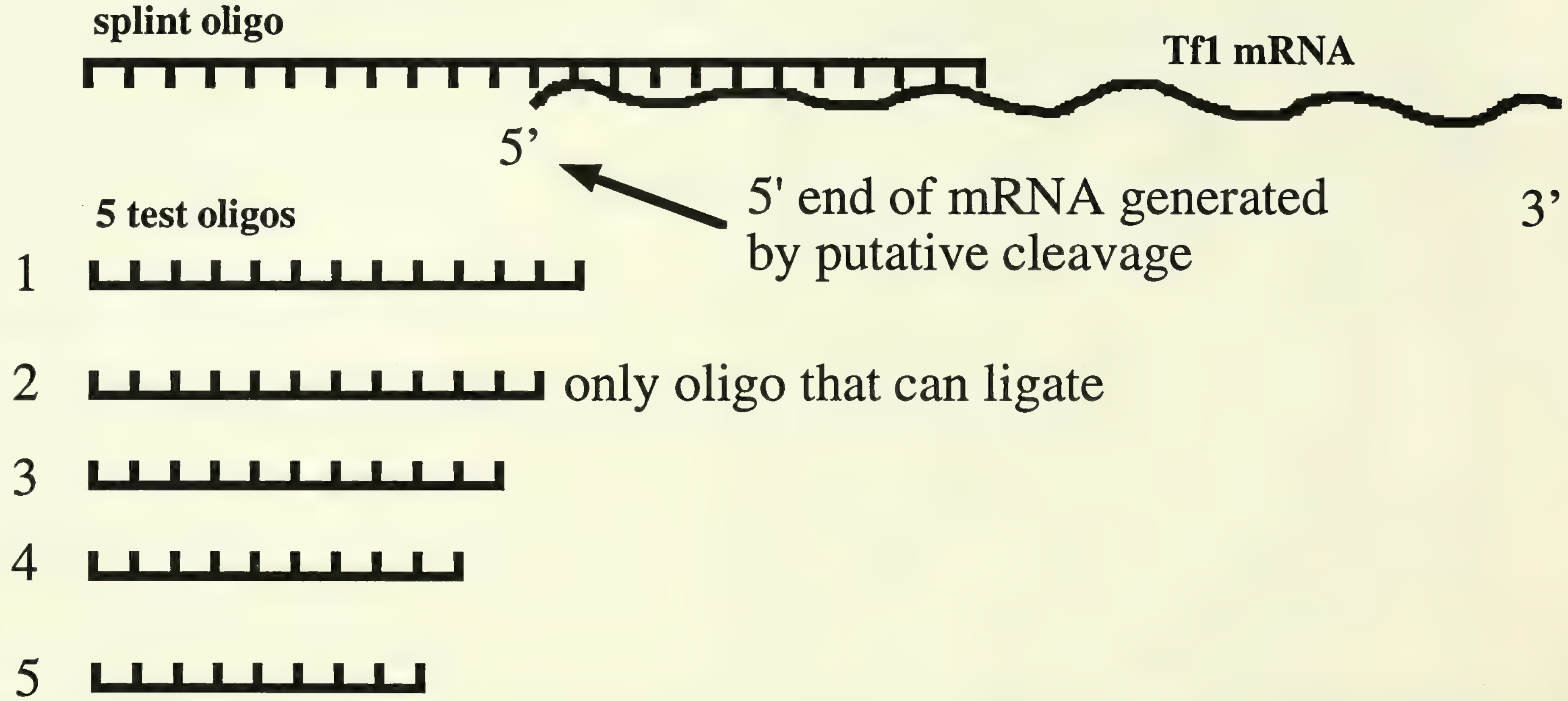

splint oligo
Tf1 mRNA
5'
3'
5' end of mRNA generated by putative cleavage
5 test oligos
1
2
only oligo that can ligate
3
4
5

Figure 2. The splinted-ligation reaction. Five different oligonucleotides were individually annealed to a splint oligonucleotide and the Tf1 mRNA. T4 ligase was only able to ligate oligo #2 to the mRNA indicating that the cleavage of the mRNA occurred between the 11th and 12th bases.

Distribution of Single Point Mutants on the Putative 5'-UTR Structure

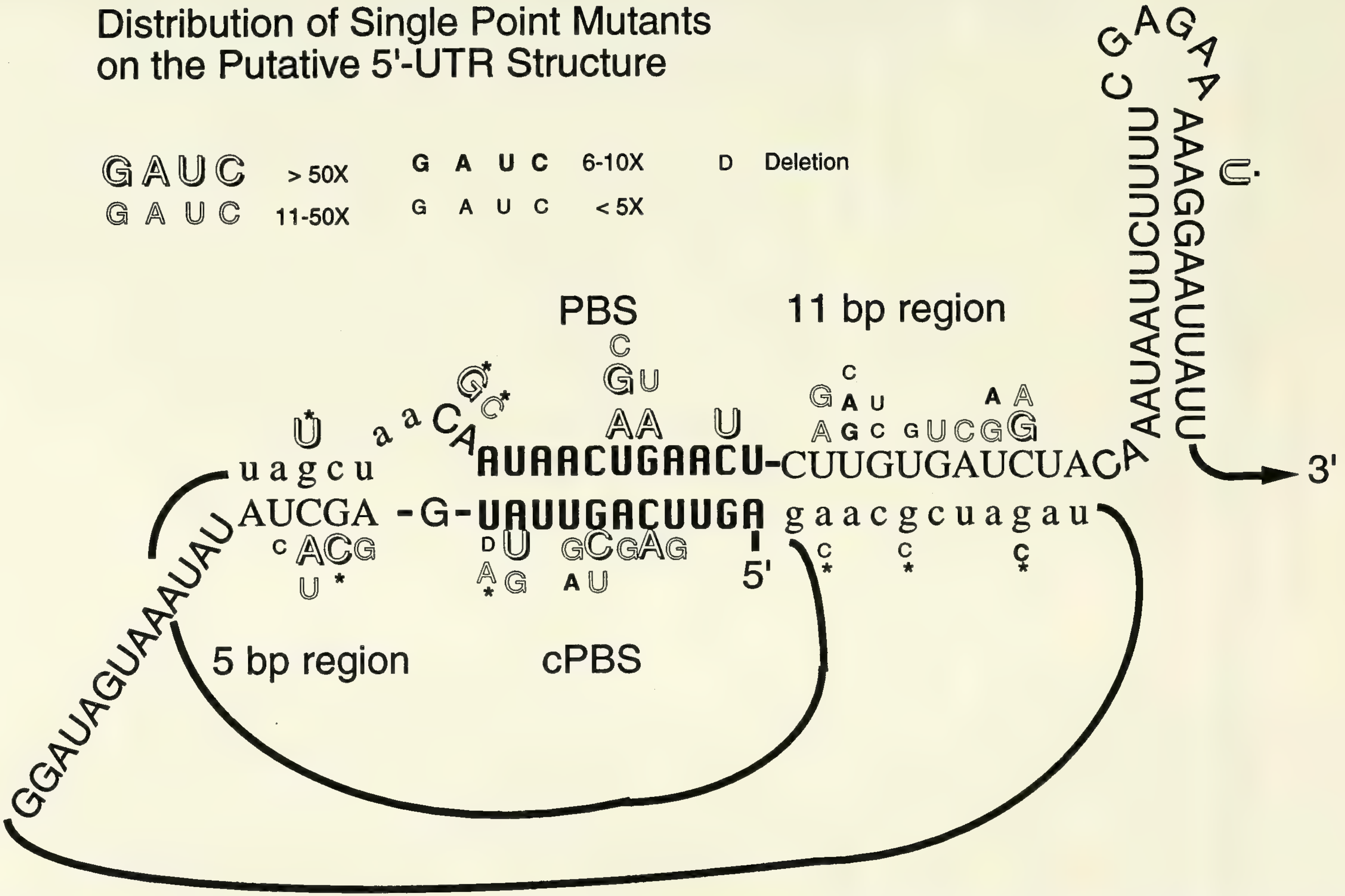


Figure 3. Mutations in the complex RNA structure. The sequence in the 5' UTR of the Tf1 mRNA has the potential to form a complex structure. Bases shown in capitol letters were subjected to random mutagenesis. The mutations isolated are shown above the RNA and the size of the letter indicates the magnitude of the transposition defect caused by the mutation.

Publications for FY96

Levin, H. L. An unusual mechanism of self-primed reverse transcription requires RNase H for cleavage of an RNA duplex, Mol and Cell Biol 1996; in press.

Atwood A, Lin J, Levin H. The retrotransposon Tf1 assembles virus-like particles with excess Gag due to specific degradation of Pol protein, Mol Cell Biol 1996;16:338-346.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01010-02 LEGR

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Eukaryotic Protein Synthesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Thomas E. Dever	Unit Head	LEGR:NICHD
Others:	Choi, Sang Ki	Visiting Fellow	LEGR:NICHD
	Kobayashi, Makiko	Visiting Fellow	LEGR:NICHD
	Lee, Joon Haeng	Visiting Fellow	LEGR:NICHD
	Lu, Jingfang	Visiting Associate	LEGR:NICHD
	Ung, Tekly	Volunteer	LEGR:NICHD

COOPERATING UNITS *(if any)*

Departments of Genetics and Entomology, University of Georgia, Athens, GA 30602 (Lois K. Miller)

LAB/BRANCH

Laboratory of Eukaryotic Gene Regulation

SECTION

Unit on Protein Biosynthesis

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.75	4.50	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The regulation of protein synthesis in mammalian cells under stress conditions as well as the regulation of GCN4-specific translation in the yeast Saccharomyces cerevisiae under amino acid starvation conditions is mediated by phosphorylation of the eukaryotic translation initiation factor (eIF)-2. We have been studying how the mammalian PKR and HRI kinases and the yeast GCN2 kinase specifically recognize and phosphorylate serine-51 on the a subunit of eIF2 to regulate translation. One approach has been to study the vaccinia virus K3L protein, a pseudosubstrate inhibitor of PKR. We have established a system to suppress the toxicity of PKR expression in yeast by co-expressing K3L. Mutational analyses have revealed that residues near the carboxyl-terminus of K3L, that are conserved in eIF2a (residues 73-83), are critical for K3L activity. The corresponding residues in eIF2a are required for proper regulation of GCN4 expression suggesting that contacts over 20 residues from site of phosphorylation are important for kinase recognition of eIF2α. The suppression of PKR toxicity in yeast has also been used to identify a novel eIF2α kinase inhibitor from the baculovirus Autographa californica. The pk2 protein of baculovirus resembles a truncated eIF2 kinase domain and we have found that when expressed in yeast pk2 can inhibit both the PKR and GCN2 kinases. Similarly, the corresponding domain of GCN2 also acts as a dominant-negative inhibitor. In an analysis of substrate specificity of the eIF2 kinases it was found that all three eIF2 kinases readily phosphorylated threonine in place of eIF2α serine-51, and PKR was able to phosphorylate tyrosine at this position as well. In addition, PKR was found to autophosphorylate on tyrosine. These results identify PKR as a dual-specificity protein kinase. Finally, we have initiated a project examining a novel yeast protein, FUN12, which resembles a bacterial translation factor that performs the same role as eIF2.

Project Description:

Objective: To understand the mechanism and regulation of protein biosynthesis in eukaryotic organisms, especially focusing on the phosphorylation of eIF2α by the yeast Saccharomyces cerevisiae GCN2 kinase and by the mammalian PKR kinase, and to understand how viruses circumvent this regulatory network.

Major Findings:

1. Mutational analysis of the vaccinia virus K3L protein.

The vaccinia virus K3L protein is a pseudosubstrate inhibitor of the eIF2a kinase PKR, a component in the mammalian antiviral defense mechanism. To increase our understanding of how the eIF2α kinases recognize their substrate we have carried out an analysis of the K3L protein. Using a yeast strain in which expression of PKR is under the control of a galactose-inducible GAL promoter we examined the ability of K3L to suppress the toxicity of PKR in yeast. High level expression of PKR is lethal in yeast, and when co-expressed K3L can suppress this toxicity. Previous work has identified both upmutants of K3L that enable it to more effectively inhibit PKR and loss-of-function mutations that block the ability of K3L to inhibit PKR. Two types of loss-of-function mutations have been identified. Point mutations introduced into the sequence 'KGYID' (residues 74-78), which corresponds to the same sequence in eIF2α (residues 79-83), abolish K3L function. In addition truncation of 6 or 7 residues from the carboxyl terminus of K3L (ending at residues 82 or 83) blocks K3L activity. These results suggest an important function for the region of K3L between residues 74 and 83 which includes the sequence KGYID that is conserved in eIF2α. One possibility is that the PKR kinase recognizes and interacts with the KGYID sequence when binding to K3L or eIF2α.

In addition to the loss-of-function alleles, upmutants of K3L have also been identified. Previously, a single strong upmutant K3L-H47R was characterized. This mutation increases the homology between K3L and eIF2α flanking the serine-51 phosphorylation site in eIF2α. In fact, the H47R mutation in K3L aligns with arginine-52 in eIF2α immediately beside serine-51. We have proposed that this mutation increases binding affinity between K3L and PKR. To test this proposal 'pull-down' assays have been performed. Wild-type K3L, the upmutant K3L-H47R, and an inactive truncated K3L protein were 'tagged' by adding six histidines to the carboxyl-terminus of the proteins. These proteins retained the same activity of the untagged forms. The three K3L proteins were co-expressed with wild-type PKR in a yeast strain carrying an eIF2α-S51A mutation. This latter mutation disrupts the autoregulation of PKR expression and so insures that all three yeast strains express equal amounts of PKR. Using a nickel resin to precipitate the his-tagged K3L protein, SDS gels followed by immunoblots were used to examine the amount of PKR that could be precipitated along with the K3L. Consistent with our model for the K3L alleles, it was found that the K3L-H47R upmutant could 'pull-down' a greater amount of PKR than wild-type K3L indicating that the K3L-H47R protein has a higher affinity for PKR. In addition, the inactive truncated K3L protein 'pulled-down' less PKR than wild-type K3L. Additional biochemical experiments demonstrated that the growth properties of strains expressing the various K3L alleles was consistent with their ability to inhibit eIF2α phosphorylation in vivo. Using isoelectric focusing gels to examine eIF2α phosphorylation it was found that the upmutant K3L-H47R more effectively inhibited phosphorylation of eIF2α than did wild-type K3L, while an inactive K3L mutant failed to inhibit eIF2α phosphorylation in vivo.

We have repeated the screen for K3L upmutants, and have identified several new candidates. Several of these new K3L upmutants contain multiple mutations, however, one mutation K3L-F36S is quite interesting. Two strains of vaccinia virus, Copenhagen and WR, are commonly studied. One of the differences between these strains is a single amino acid change in K3L. This change is at residue 36, where Copenhagen encodes serine and WR encodes phenylalanine. Our identification of serine-36 as an upmutation for K3L from the WR vaccinia virus raises the possibility that the Copenhagen strain of vaccinia virus may more effectively down-regulate PKR during viral infection.

The analysis of K3L has provided clues into the motifs that eIF2 kinases recognize on their substrate. We have further exploited this yeast system to provide insights into the structural elements in the kinases that facilitate substrate recognition. A K3L-H47R expression vector was introduced into a yeast strain. This strain is resistant to wild-type PKR because K3L is an effective inhibitor of PKR toxicity. To increase our understanding of how K3L inhibits PKR and to map potential regions of contact between PKR and K3L (or eIF2α) a screen for PKR mutants resistant to K3L was performed. A PKR gene under the control of a galactose-inducible promoter was randomly mutated using low fidelity PCR or by passage through an E. coli strain deficient in repair mechanisms. A pool of mutant PKR alleles was introduced into the yeast strain carrying the K3L expression vector and colonies that grew slowly or not at all when PKR expression was induced were identified. Following confirmation of the results, the PKR plasmids were isolated and sequenced. So far six independent PKR mutant alleles have been identified. One of these alleles contains the single amino acid substitution I378T. This mutation maps in subdomain VI of protein kinases, and based on the crystal structure of the cAMP-dependent protein kinase is predicted to be in close proximity to the substrate binding site of the kinase.

2. Examination of substrate specificity of the eIF2α kinases.

Most protein kinases can be classified into one of two classes either serine/threonine specificity or tyrosine specificity. However, a few kinases have been identified with dual specificity that will phosphorylate both tyrosine and serine/threonine residues. To date the only known physiologically relevant substrate of the eIF2α kinases is eIF2α, however recent reports suggest that PKR may have alternative substrates. While the eIF2α kinases have only been reported to phosphorylate serine residues the alternative phospho-acceptors threonine and tyrosine have not been examined. Intriguingly, the mouse PKR kinase was originally identified in a screen for tyrosine kinases. However, in this study there was no evidence that PKR was phosphorylated on or that PKR could phosphorylate other proteins on tyrosine residues. To further investigate the substrate specificities of the eIF2α kinases and as a possible means to examine the differences between the serine/threonine and tyrosine kinases we mutated the serine-51 residue in eIF2α to threonine and tyrosine. We then used genetic and biochemical tests to examine the ability of the three eIF2 kinases (GCN2, PKR and HRI) to phosphorylate these alternative residues. Yeast cells expressing the eIF2α-S51T allele were practically indistinguishable from wild-type cells. When GCN2 or HRI was introduced into the strain the cells were able to grow under amino acid starvation conditions, indicating that both GCN2 and HRI can phosphorylate the eIF2α-S51T protein. In addition, high level expression of PKR in the eIF2a-S51T strain was lethal again demonstrating that the eIF2α kinases can phosphorylate a threonine residue. These results also demonstrate that phosphothreonine can substitute for phosphoserine in the regulation of translation by phosphorylated eIF2. Biochemical analyses using isoelectric focusing gels have confirmed that all three eIF2 kinases can phosphorylate the eIF2α-S51T protein.

When serine-51 was replaced with tyrosine initial results suggested that tyrosine was not a substrate. Yeast strains expressing GCN2 and carrying the eIF2α-S51Y allele are sensitive to amino acid starvation conditions and high level expression of PKR in the eIF2α-S51Y strain is not lethal. Both of these results are consistent with the eIF2α kinases being unable to phosphorylate tyrosine residues. However, the strains expressing PKR to high levels could grow under amino acid starvation conditions suggesting that PKR may be phosphorylating the tyrosine residue and inducing GCN4 expression. To directly analyze phosphorylation of eIF2α-S51Y, isoelectric focusing analysis was performed. A signal consistent with phosphorylation of eIF2α was observed in strains expressing PKR or HRI and either wild-type eIF2α or eIF2α-S51Y, but not eIF2α-S51A. In addition, the phosphorylation of eIF2α-S51Y was dependent on the wild-type kinase and was not observed with an inactive mutant kinase. To confirm that the eIF2α-S51Y protein was indeed phosphorylated on tyrosine the immunoblot from the isoelectric focusing gel was probed with anti-phosphotyrosine antibodies. A signal was specifically detected in the strains expressing PKR or HRI and eIF2α-S51Y, but not wild-type eIF2α. The finding that PKR and HRI can phosphorylate a tyrosine residue in vivo raises the possibility that alternate substrates for PKR or any of the eIF2α kinases may be phosphorylated on tyrosine residues rather than exclusively on serine residues.

The ability to phosphorylate serine/threonine and tyrosine residues identifies PKR as a dual specificity protein kinase. A common feature of a number of dual specificity protein kinases is the ability to autophosphorylate on tyrosine. As mentioned previously, mouse PKR was identified in a screen for tyrosine kinases, however, no phosphotyrosine was identified on the mouse PKR. We examined the possibility that human PKR could autophosphorylate on tyrosine by performing immunoblotting and immunoprecipitation experiments. Human PKR or an inactive mutant PKR-K296R were expressed in E. coli and immunoblotting experiments were performed with anti-PKR or anti-phosphotyrosine antibodies. The wild-type PKR was detected using both antibodies while the mutant kinase was only detected by the anti-PKR antibodies. Consistent with these results, wild-type PKR expressed in E. coli or yeast could be immunoprecipitated using anti-phosphotyrosine antibodies. To confirm that PKR was phosphorylated on tyrosine, phosphoamino acid analysis was performed. In a collaboration with Dr. Patrick Romano, phosphoamino acid analysis was performed on PKR expressed in yeast. PKR isolated from in vivo labelled yeast cells was found to contain phosphoserine, phosphothreonine and phosphotyrosine. Finally, we examined the authentic PKR expressed in mammalian cells. We found that we could immunoprecipitate PKR from interferon-treated HeLa cells using anti-phosphotyrosine antibodies. These results demonstrate that PKR is a dual specificity protein kinase that can autophosphorylate on tyrosine when expressed in E. Coli, yeast, or mammalian cells.

3. Identification and characterization of a baculovirus inhibitor of eIF2α kinases.

Many viruses have evolved ways to counteract the antiviral defense mechanisms of eukaryotic cells. One of the defense mechanisms cells employ is phosphorylation of eIF2α by the PKR kinase. As described above, vaccinia virus expresses the K3L protein, a pseudosubstrate inhibitor of PKR. Other viruses express different protein or RNA inhibitors of PKR and still others activate latent cellular inhibitors of PKR or even degrade PKR. When the sequence of the baculovirus Autographa californica was reported late in 1994 an open reading frame designated ORF123 encoding a truncated protein kinase termed pk2 was identified. The amino acid sequence of pk2, though truncated, was most similar to the eIF2α kinase family. Since many viruses encode inhibitors of the eIF2α kinases, we reasoned that pk2 might be an inhibitor of PKR. To test this hypothesis the pk2 gene was isolated from baculovirus using the PCR and the gene was inserted into the vector pEMBLyex4. Introduction of the pk2 expression plasmid into the yeast strain that expresses PKR under the control of the GAL promoter revealed that pk2 could suppress the toxicity of PKR in yeast. Not only could pk2 suppress PKR toxicity, but pk2 was also able to suppress both wild type and hyperactive alleles of the yeast eIF2α kinase GCN2. These results demonstrate that pk2 is an eIF2α kinase inhibitor, and since baculovirus is an insect virus this is the first indication that insects may also use phosphorylation of eIF2α as a means to block viral infection.

We have continued this analysis to gain insights into how pk2 inhibits the eIF2 kinases. Immunoblot analyses have determined that expression of pk2 does not block PKR expression in yeast. However, using isoelectric focusing gels to examine eIF2α phosphorylation, we have found that expression of pk2 lowers the level of eIF2α phosphorylation by both PKR and GCN2. These results are consistent with pk2 directly inhibiting the eIF2α kinases. To further test how pk2 inhibits the eIF2α kinases we have tested the ability of mutant kinases to block pk2 activity. This experiment was designed to test the model that pk2 inhibits the kinases by directly contacting the kinase as opposed to sequestering the substrate eIF2. Expression of pk2 in yeast was found to inhibit the yeast eIF2 kinase GCN2 resulting in a sensitivity to amino acid starvation conditions. Overexpression of a catalytically inactive PKR-K296R kinase has no effect on GCN2 activity. However, overexpression of PKR-K296R suppressed the ability of pk2 to inhibit GCN2. The favored interpretation of this result is that the overexpressed PKR-K296R protein was able to sequester pk2 and free GCN2 to phosphorylate eIF2α.

The pk2 protein is similar to the carboxyl-terminal half of an eIF2α kinase domain. This raised the possibility that the corresponding fragment from an authentic eIF2α kinase domain may also act in a dominant negative manner. The amino acid sequences from GCN2 and pk2 were aligned and oligonucleotides were identified to amplify the corresponding region of the GCN2 kinase using the PCR. Following cloning into pEMBLyex4, the truncated gcn2 kinase domain was introduced into various strains. High level expression of the truncated gcn2 kinase domain was found to inhibit the toxicity associated with the hyperactive GCN2^c kinases and thus reveal that this region of an eIF2α kinase can act in a dominant negative manner. Similar experiments with truncated versions of the PKR and HRI kinase domains failed to detect any dominant-negative activities suggesting that the dominant-negative activity may have unique and stringent requirements.

To study the function of pk2 in the baculovirus we initiated a collaboration with Dr. Lois K. Miller at the University of Georgia. Deletion of pk2 from baculovirus has no effect on the ability of the virus to infect cells from two different common hosts (S. frugiperda and T. ni). However, these negative results may reflect the fact that we do not know the proper conditions to induce expression of an eIF2α kinase in insect cells. Alternatively, the pk2 protein may be important for the infection of alternative hosts of baculovirus. To determine if pk2 can protect the virus from an eIF2 kinase we introduced the human PKR kinase into S. frugiperda cells and then infected with wild-type or pk2-deleted baculovirus. Expression of PKR inhibited infection by the pk2-deleted virus by about 50%, and expression of pk2 in trans was able to restore viral infectivity. These results demonstrate that the pk2 protein can protect baculovirus from the negative effects of an eIF2 kinase, and they raise the possibility that the pk2 protein may be important for baculovirus infection of other hosts or under special conditions where eIF2 kinases are expressed.

4. Molecular genetic analysis of the yeast FUN12 gene and characterization of its role in translation.

The sequencing of the yeast genome revealed the presence on chromosome I of an open reading frame encoding a protein showing significant amino acid sequence similarity to the bacterial translation factor IF2. The function of IF2 in bacterial cells is to deliver the initiator Met-tRNA to the ribosome to enable the synthesis of polypeptide chains. In eukaryotic cells this function is performed by the three subunit factor eIF2. The identification of this bacterial-like translation factor in yeast suggested two possibilities. First, the yeast IF2-like factor, termed FUN12, could function in the specialized translation system of the mitochondria which more closely resembles bacterial translation. Alternatively, FUN12 may be part of an alternative translation mechanism operating in eukaryotic cells. Several findings argue against the first possibility. The yeast mitochondrial IF2 protein has previously been identified and is not encoded by FUN12. In addition, we have found that disruption of the FUN12 gene does not impair respiration as would be expected of a factor required for mitochondrial protein synthesis. However, disruption of FUN12 does severely impair the growth rate of a yeast strain.

To identify the role of FUN12 in the cell we have characterized the yeast strains deleted for FUN12. One of the major distinguishing features between bacterial and eukaryotic translation is the binding of the ribosome to the mRNA. In bacterial cells the ribosome is directed to bind to the mRNA through a sequence termed the Shine-Delgarno box that is found adjacent to the initiating AUG codon. Following or concurrent with ribosome binding to the mRNA the Met-tRNA$_i^{Met}$ is bound to the ribosome. In eukaryotic cells the Met-

$tRNA_i^{Met}$ is believed to be delivered to the ribosome prior to the ribosome binding at the mRNA 5' end and scanning to the initiating AUG codon. In eukaryotic cells it is believed that eIF2 functions to deliver the Met-$tRNA_i^{Met}$ to free 40S ribosomal subunits, however, alternate forms of translation initiation in eukaryotic cells may require a different factor. Two alternate mechanisms of translation in eukaryotic cells are re-initiation and internal initiation. In re-initiation the Met-$tRNA_i^{Met}$ must be delivered to a ribosome already bound to mRNA, while in internal initiation the ribosome binding to the mRNA is not directed by the 5' end of the mRNA but instead is directed to an internal region of the mRNA. These alternative mechanisms of translation initiation in mammalian cells more closely resemble the bacterial mechanism of translation and may be targets for the FUN12 factor in eukaryotic cells. To test this possibility we have examined the effect of deleting the FUN12 gene on reinitiation and internal initiation in yeast cells.

In yeast the ideal system to analyze reinitiation is the regulation of GCN4 expression. In response to amino acid starvation GCN4 expression is derepressed ca. 9-10-fold. This derepression is the result of altered efficiency of translational reinitiation on the GCN4 mRNA. In preliminary experiments we have found that disruption of FUN12 blocks the derepression of GCN4 expression under starvation conditions. To examine internal initiation in yeast we have relied on two independent assays. First, we have examined the maintenance of the yeast double-stranded RNA virus-like particles L-A and M. Yeast cells can support the replication of both L-A and M viruses. These viruses are maintained as cytoplasmic elements that utilize the yeast cells' translational machinery for viral protein synthesis. The L-A and M RNAs are uncapped, and therefore must be translated using an alternative cap-independent mechanism. To determine if FUN12 was involved in the translation of these RNAs we examined the effect of deleting FUN12. In wild-type strains where both L-A and M RNAs are translated efficiently the M virus expresses a toxin called 'killer' that kills yeast cells not expressing M proteins. The M virus is in fact a satellite virus of L-A and high levels of L-A virus are required to support the maintenance of M in yeast cells. We examined the effects of deleting FUN12 on the maintenance of the L-A and M viruses in yeast. Yeast cells deleted for FUN12 no longer express the killer toxin and fail to maintain the M virus. This result suggests that FUN12 is required for the translation of the L-A and/or M viruses, and thus may function in cap-independent translation.

A second assay was also developed to examine internal initiation of translation in yeast. The traditional reporters for internal translation initiation examine the ability of mRNA sequences to support the translation of the second open reading frame (ORF) of a bicistronic mRNA. In eukaryotic cells the second ORF of a bicistronic mRNA is usually poorly expressed. However, when mRNA sequences that support internal ribosome binding are inserted between the two ORFs of a bicistronic mRNA translation of the second ORF is increased. Internal initiation of translation has been observed in mammalian cells on both viral (polio and encephalomyocarditis) and cellular (eIF4G, BiP) mRNAs. The yeast homolog of mammalian eIF4G is TIF4631. Similar to the mammalian gene, the TIF4631 mRNA contains a large number of upstream ORFs, which would be expected to interfere with the normal cap-dependent mechanism of translation. We therefore tested to see if the yeast TIF4631 mRNA could support internal initiation in yeast. A polycistronic yeast mRNA derived from the GCN4 gene was used as a template. A GCN4-lacZ allele in which only the third and fourth uORFs of GCN4 are intact expresses very low levels of β-galactosidase. When the leader region of the TIF4631 mRNA was inserted between ORF4 and the GCN4-lacZ AUG codon, the β-galactosidase activity increased 6- to 30-fold. These results indicate that yeast cells can support internal initiation, and that the mRNA leader region of the eIF4G gene from mammalian or yeast cells can function to direct internal ribosome binding and translation initiation. When the same GCN4-lacZ construct containing the TIF4631 leader was assayed in a fun12 deletion strain β-galactosidase activity dropped 2- to 4- fold. These results suggest that FUN12 plays an important role in the internal translation initiation mechanism. The FUN12 protein is the first trans-acting factor that has been identified to function in internal initiation and reinitiation. These results take on added significance with our identification of an expressed sequence tag (EST) from mouse that bears striking homology to FUN12, raising the possibility that this protein is conserved in humans as well.

Proposed Course of Project:

1. eIF2α mutants defective for translational regulation. We plan to resume our analysis of eIF2α mutants identifying alleles that fail to properly induce GCN4 expression under amino acid starvation conditions. Previously, we identified over 100 different mutations in eIF2α that block translational regulation in yeast by the GCN2 protein kinase. GCN2 is a yeast kinase that is activated under amino acid starvation conditions and phosphorylates eIF2α on serine-51. The phosphorylation of eIF2α by the GCN2 kinase in yeast causes a modest inhibition of general translation, but also specifically stimulates the translation of GCN4 mRNA. The increased synthesis of GCN4 enables strains to grow under amino acid starvation conditions. In previous experiments we have identified numerous mutations in eIF2α that block the ability of cells to grow under amino acid starvation conditions. These mutations may either block the ability of GCN2 to phosphorylate eIF2α or they may block the regulation of translation by phosphorylated eIF2. In the previous work we focused our mutational analysis of eIF2α to the residues immediately flanking the serine-51 residue of eIF2α and to the residues conserved between eIF2α and K3L. In this latter class we have especially focused on the "KGYID' sequence. Our results indicated that residues E49 and L50 in the sequence $SELS_{51}RRR$ were important for proper regulation of GCN4 expression by the GCN2 kinase. Any substitution at E49 and at least 10 different substitutions at L50 blocked regulation of GCN4 expression. Many substitutions in the sequence 'KGYID' also disrupted the regulation of GCN4 expression by GCN2.

We will continue our analysis of these mutants and try to distinguish between the two mechanisms by which the mutations may be altering regulation. The eIF2α mutants will be screened in a strain carrying a mutation in the eIF2B complex to see if any of the eIF2α mutants can suppress a mutation in eIF2B. The eIF2B complex is inhibited by phosphorylated eIF2 and mutations have been identified in eIF2B that mimic the effects of eIF2 phosphorylation. If the eIF2α mutants block the inhibition of eIF2B by phosphorylated eIF2 they may also suppress a mutation in eIF2B. We will screen the eIF2α mutants for the ability to suppress a mutation in the GCN3 subunit of eIF2B. The mutations in eIF2α may instead block the phosphorylation of eIF2α by GCN2. These mutations would then identify the residues GCN2 utilizes to recognize eIF2α and phosphorylate serine-51. We will examine the phosphorylation of serine-51 in the mutant proteins using the isoelectric focusing gels. While only a single mutation (L50P) has been identified that severely impairs phosphorylation, a few mutations have been identified that appear to reduce phosphorylation. By constructing chimeric eIF2α proteins containing combinations of mutations that alone modestly impair phosphorylation, we hope to obtain additional alleles that are severely impaired for phosphorylation by GCN2 in vivo. The eIF2α alleles that are defective for phosphorylation by GCN2 in vivo will also be examined both in vivo and in vitro with PKR. The ability of these alleles to suppress the toxicity of high level PKR expression in yeast will be monitored, and the ability of PKR to phosphorylate these proteins will be examined both in vivo (using the isoelectric focusing gels) and in vitro. The in vitro kinase assays will employ either recombinant PKR or PKR immunoprecipitated from yeast and the substrate will be recombinant eIF2α alleles expressed using bacterial expression systems. Alleles of eIF2α that are defective for phosphorylation by PKR will be used in a reversion analysis to identify PKR mutants with altered substrate specificity. Pools of mutant PKR genes will be introduced into yeast cells expressing the mutant eIF2α alleles, and PKR mutants that restore phosphorylation will be identified as causing a slow-growth phenotype or increased resistance to amino acid starvation conditions.

A new screen will also be initiated to identify complementing pairs of eIF2α and PKR mutations. A pool of eIF2α mutants that block translational regulation will be introduced into a yeast strain lacking an eIF2 kinase. A pool of randomly mutated PKR or GCN2 alleles will be introduced into a second strain also lacking an eIF2 kinase, but expressing an eIF2α-S51A protein, The two strains will be mated and diploids that restore translational regulation will be identified. These candidate diploids contain mutant alleles of eIF2α and either PKR or GCN2 that fail to regulate translation with the partner wild-type protein, but when paired with the particular mutant partner can restore regulation. These complementing alleles are expected to help define recognition elements and points of contact between the eIF2α kinases and their substrate.

2. Analysis of the vaccinia virus K3L protein. We will continue our screen to identify PKR mutants that are resistant to K3L. PKR mutants resistant to K3L-H47R will be identified as causing a slow-growth or no-growth phenotype in a strain expressing K3L-H47R. As described previously this strain grows well when wild-type PKR is expressed because K3L can inhibit wild-type PKR. The PKR mutants that are resistant to K3L-H47R will be examined to determine their resistance to the various K3L upmutants uncovered in the other screens. The identification of PKR mutants resistant to K3L will help identify the substrate binding region in PKR; and through the examination of the various K3L and PKR mutants we may be able to map the interacting surfaces in PKR and K3L. It is predicted that the PKR mutants resistant to K3Lwill have a lower affinity for K3L. To analyze the binding between PKR and K3L we will use co-immunoprecipitation assays, and the yeast two-hybrid system. These results should help identify how PKR and the other eIF2α kinases interact with their authentic substrate eIF2α, and they should provide important insights into the mechanism of pseudosubstrate inhibition of protein kinases.

We are planning to further analyze our K3L and PKR mutants in a mammalian system. In collaboration with Dr. Bertram Jacobs at Arizona State University, we plan to examine the ability of various upmutants and loss-of-function mutants of K3L to substitute for wild-type K3L in a viral infection assay. The mutant K3L genes will be introduced into vaccinia virus in place of the authentic K3L gene and the interferon sensitivity of the chimeric virus will be determined. In addition, we plan to examine the ability of the chimeric vaccinia viruses to protect other viruses, such as EMC, from the antiviral effects of interferon. In a related set of experiments we plan to express in mammalian cells one of the PKR mutants that is resistant to K3L and determine its effects on viral infection. These experiments will help us address the anti-viral role of PKR and they may provide new insights into ways to aid and improve the anti-viral response in mammalian cells.

3. Analysis of the baculovirus pk2 protein. A primary goal for this study is to determine how pk2 inhibits the eIF2α kinases. Our genetic results suggest that pk2 directly interacts with the eIF2 kinases, probably by forming an inactive dimer. To provide physical evidence to support this model co-immunoprecipitation experiments will be performed. The pk2 protein has been modified by the addition of a c-myc epitope at its carboxyl-terminus. This epitope-tagged protein is still functional as an inhibitor of PKR and GCN2. Using anti-myc antibodies we will immunoprecipitate pk2 and examine the ability to co-precipitate PKR or GCN2. If co-precipitation experiments fail we may use the yeast two-hybrid system to examine interactions between pk2 and either PKR or GCN2.

To map the region of pk2 required to inhibit PKR, truncated alleles will be constructed using the PCR. These alleles will then be tested for their ability to inhibit PKR or GCN2. In addition we are planning a random mutational approach to identify important residues in pk2. The pk2 gene will be randomly mutated using low fidelity PCR and alleles that are either severely impaired for function or more active will be identified. High level expression of pk2 is toxic in yeast and we are interested to see if we can separate pk2 toxicity from its ability to inhibit the eIF2α kinases. These mutational analyses should help to identify how pk2 functions as an inhibitor and they may provide insights into the mechanism of dimerization that has been proposed for at least some of the eIF2 kinases. Finally, the slow-growth phenotype observed when pk2 is expressed to high levels in yeast cells suggests that pk2 may inhibit other kinases in yeast. We will screen for high copy-number suppressors of pk2 toxicity in yeast to identify other potential targets of pk2. These experiments are particularly intriguing because they may help identify additional roles for pk2 during baculovirus infections.

4. Molecular analysis of yeast FUN12 and molecular genetic analysis of internal initiation of translation in yeast.

We will continue to study the role of FUN12 in cellular protein synthesis. A primary goal of the FUN12 research is to demonstrate that the FUN12 protein can bind Met-tRNA$_i^{Met}$ as expected for an IF2-like protein. Following our identification of FUN12 and our determination that FUN12 functions in cellular translation we reviewed the literature to see if any mammalian IF2-like factors have been reported. Interestingly, a protein named eIF2A has been described that possesses properties consistent with an IF2-like or FUN12 protein. We suspect that eIF2A may be mammalian FUN12 (and may be encoded by the mouse est cDNA mentioned earlier). This hypothesis is leading us to test FUN12 for the ability to bind Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit in an AUG-dependent manner. In these filter-binding experiments we will identify the requirements for FUN12 to bind Met-tRNA$_i^{Met}$.

Two types of molecular genetic analyses are planned on FUN12. First, we will screen for dosage-dependent suppressors of the slow-growth phenotype of a fun12 deleted yeast strain. Deletion of FUN12 causes a severe slow-growth phenotype in one of our strain backgrounds and we will introduce a high copy-number yeast DNA library and screen for faster growing colonies. In this screen we hope to identify either targets of FUN12 or other factors that function in the alternate translation mechanisms with FUN12, In the second screen, the FUN12 gene will be randomly mutated and the mutants will be screened to identify any temperature-sensitive (ts$^-$) alleles or alleles that alter GCN4 translation. Biochemical analysis of FUN12 ts$^-$ mutants should help define the step of translation at which FUN12 acts. In addition to random mutants, site-directed mutations will be introduced into the FUN12 GTP-binding motif. Mutations in this region of bacterial IF-2 have been identified that lead to a slow-growth phenotype, we will examine the effects of the corresponding mutations in yeast FUN12. These suppressor and mutational analyses of FUN12 should help to define the role of FUN12 in translation, and should provide new insights into the alternate mechanisms of translation initiation.

We will continue our analysis on internal initiation of translation by further characterizing the TIF4631 sequences that can direct internal initiation. Currently, a 500-bp fragment from the TIF4631 mRNA has been shown to mediate internal initiation. We will analyze smaller pieces of this fragment in our system to determine a minimal sized fragment that can support internal initiation. In addition to identifying the sequence requirements for internal initiation, we will also screen for trans-acting factors that can promote internal initiation. First, we will examine the effects of known translation factor mutations on our reporter construct. The TIF4631-GCN4-lacZ reporter construct will be introduced into various yeast strains containing mutant alleles of known translation factors, and β-galactosidase assays will be used to analyze the effects of the mutations on internal initiation. In a second screen we will identify genes, which when overexpressed, can facilitate internal initiation. A TIF4631-GCN4 allele will be used to complement a GCN4-deleted yeast strain. This allele confers a partial resistance to amino acid starvation conditions. A high copy-number yeast DNA library will be introduced into this strain and transformants which grow better under starvation conditions will be identified. Among these selected transformants are expected to be plasmids encoding factors that function in or stimulate internal initiation. In addition to the random screen we will directly test several candidate genes, like FUN12, in this assay. Finally, we plan to directly examine internal initiation on the TIF4631 gene, as opposed to our chimeric constructs with GCN4. These molecular genetic analyses should provide important new insights into the mRNA sequence and trans-acting factor requirements for internal initiation of translation.

Significance to Biomedical Research and the Program of the Institute.

The eIF2α kinases play a key role in the antiviral and stress responses of mammalian as well as yeast cells. The ability of cells to alter gene expression under stress conditions is essential to the survival of the organism. In addition, the genetic characterization of substrate recognition by the eIF2α kinases should provide insights into the mechanism of substrate recognition by other protein kinases. Increasing our understanding of viral inhibitors of the eIF2α kinases will not only provide insights into the molecular mechanisms regulating these kinases, but may also identify ways to defeat these viral defense mechanisms. Finally, the genetic characterization of internal initiation of translation and the analysis of the role of FUN12 in alternate mechanisms of translation will provide new insights into the mechanisms of gene expression used by cells and may provide new insights into the ways cells can reprogram their gene expression during development or cellular stress conditions.

Publications:

Dever TE, Yang W, Astrom S, Bystrom AS, Hinnebusch AG. Modulation of $tRNA_i^{Met}$, eIF2 and eIF2B expression shows that GCN4 translation is inversely coupled to the level of the $eIF2\bullet GTP\bullet Met\text{-}tRNA_i^{Met}$ ternary complexes, Mol. Cell. Biol. 1995;15:6351-6363.

Dever TE. Analysis of eIF2α kinases in yeast. In: Martin R, ed. Methods in Molecular Biology: Protein Synthesis: Methods and Protocols. Totawa, NH: Humana Press Inc. (In Press).

Dever TE. Using GCN4 as a reporter of eIF2α phosphorylation and translational regulation in yeast, Methods (In Press).

LME

NICHD Annual Report
October 1, 1995 to September 30, 1996

Laboratory of Molecular Embryology

The Laboratory has continued to make highly significant progress in elucidating the molecular mechanisms responsible for the differentiation of germ cells in vertebrates, for establishing totipotency in the fertilized egg and for the subsequent differentiation of distinct cell lineages during early vertebrate development. The molecular basis of cellular determination during embryogenesis is a central problem of cell biology. The establishment and maintenance of stable differentiated states is essential to the development of an organism. When such controls break down, the cell may revert to a totipotent state leading in turn to malignancy.

DEVELOPMENTAL REGULATION OF DIFFERENTIAL GENE EXPRESSION-CHROMOSOMAL STRUCTURE AND FUNCTION

The differentiated cell contains constellations of active and inactive genes. Our research has contributed to the understanding of how both of these states are established and coexist in the eukaryotic nucleus. During gametogenesis and embryogenesis active chromatin can become repressed, and repressed chromatin activated reversibly. The reconstruction and experimental manipulation of these events demonstrate the interplay between chromosomal structure and specific transcription factors in controlling differential gene expression during vertebrate development.

GENE REGULATION DURING EARLY EMBRYOGENESIS

Remodeling of somatic nuclei in *Xenopus* egg extracts leading to the acquisition of transcriptional competence

Classical nuclear transplantation experiments indicate the capacity of differentiated somatic nuclei to 'dedifferentiate' in an egg environment and acquire pluripotence sufficient to drive the development of a swimming tadpole. We have examined the molecular mechanisms responsible for the remodeling of entire somatic erythrocyte nuclei in Xenopus laevis egg cytoplasm. These transitions in chromosomal composition are associated with the capacity to activate new patterns of gene expression and the reacquisition of replication competence. Somatic linker histone variants, H1 and H1° are rapidly released from chromatin in egg cytoplasm, whereas the oocyte specific linker histone B4 and HMG1 are efficiently incorporated into remodeled chromatin. Core histones H2A and H4 in the somatic nucleus are phosphorylated during this remodeling process. These transitions recapitulate the chromosomal environment found within the nuclei of the early Xenopus embryo. We determine that phosphorylation of somatic linker histone variants is not essential for their release from chromatin, nor is competition with cytoplasmic stores of linker histone B4 and HMG1 essential for their release. We find that the molecular chaperone nucleoplasmin does have an essential role in the selective removal of linker histones from somatic nuclei. For Xenopus erythrocyte nuclei, this disruption of chromatin structure leads to the transcriptional activation of oocyte-specific genes. Our results provide a molecular explanation for the remodeling of chromatin in Xenopus egg cytoplasm, and indicate the capacity of molecular chaperones to independently disrupt a natural chromosomal environment thereby facilitating the transcription process.

CHROMATIN STRUCTURE AND FUNCTION

A new model for the nucleosome

Histone-DNA contacts within a nucleosome influence the function of trans-acting factors and the molecular machines required to activate the transcription process. We have probed the internal architecture of a positioned nucleosome using photoactivatable cross-linking reagents to determine the placement of histones along the DNA molecule. We find that the winged-helix domain of the linker histone is located inside the gyres of DNA that also wrap around the core histones. The preferential association of the linker histone to one side of the nucleosome generates an asymmetric particle that may impart a directionality to the folding of the chromatin fiber. This might be propagated by a polar head-to-tail arrangement of linker histone molecules along the nucleosomal array or chromatin fiber. The proposed orientation of the winged helix domain is also likely to favor interaction of the extended basic C-terminal tail of the linker histone with the linker DNA. Charge neutralization of the phosphodiester backbone of linker DNA by the C-terminal tail has a major role in chromatin compaction. Moreover the removal of the linker histone away from the dyad axis of the nucleosome would eliminate the need for the DNA at the entry and the exit of the nucleosome to come into close proximity. This also eliminates a central requirement of certain models of the chromatin fiber that have the linker histone uniquely localized at the center of the fiber.

Our model of the nucleosome provides a simple explanation for the influence of linker histones on transcription and nucleosome positioning. Histone H1 and H5 have been shown to direct the exact DNA sequences wrapped by the histone octamer. These linker histones also restrict nucleosome mobility. Since linker histones resemble other winged helix proteins, interactions with the major groove of DNA as suggested here would confer sequence selective recognition. Transcription factors of the winged helix class such as HNF3 have already been shown to position nucleosomes. Removal of histone H1 as seen on transcriptional activation of inducible promoters would remove a major positional signal for chromatin organization, and the resulting mobility of histone octamers with respect to DNA sequence could greatly facilitate transcriptional activation.

Differential association of HMG1 and linker histones B4 and H1 with nucleosomal DNA: structural transitions and transcriptional repression

The high mobility group proteins 1 and 2 (HMG1/2) and histone B4 are major components of chromatin within the nuclei assembled during the incubation of Xenopus sperm chromatin in Xenopus egg extract. To investigate their potential structural and functional roles, we have cloned and expressed Xenopus HMG1 and histone B4. Purified histone B4 and HMG1 form stable complexes with nucleosomes including Xenopus 5S DNA. Both proteins associate with linker DNA and stabilize it against digestion with micrococcal nuclease, in a similar manner to histone H1. However, neither histone B4 nor HMG1 influence the DNase I or hydroxyl radical digestion of DNA within the nucleosome core. HMG1/2 and histone B4 have a shared structural role in organizing linker DNA in the nucleosome.

We have examined further the structural and functional consequences of incorporating either histone H1, histone B4 or HMG1 into a synthetic dinucleosome containing two 5S rRNA genes. We found that all three proteins bind to linker DNA, stabilizing an additional 20 bp from micrococcal nuclease digestion and restrict nucleosome mobility. Histone H1 has the highest-affinity interaction with the dinucleosome; histone B4 and HMG1 associate with significantly reduced affinities. We found that histone H1 binds to the dinucleosome template with a dissociation constant (K_D) of 7.4 nM, whereas the K_D is 45 nM for histone B4 and 300 nM for HMG1. The K_Ds for the interaction of these proteins with naked DNA are 18 nM for H1, 80 nM for B4 and 300 nM for HMG1. The differences in association of these proteins with the dinucleosome are reflected in the efficiency with which the different proteins repress transcription from the 5S rRNA genes. Thus, although all three proteins can contribute to the organization of chromatin, the stability of the structures they assemble will vary. Our results provide a molecular explanation for the transcriptional promiscuity of Xenopus early embryonic chromatin, which is enriched in HMG1 and linker histone B4, but deficient in histone H1.

Nucleosome assembly on human disease genes

Expansions of CTG trinucleotide repeat sequences are now associated with several human genetic diseases. These include myotonic dystrophy, spinal and bulbar muscular atrophy, Huntington's disease, spinocerebellar ataxia, and dentatorubral-pallidoluysian atrophy. The CTG trinucleotide repeats are found associated with certain genes whose products are necessary for correct cell function. Aberrant gene expression or the synthesis of a mutant protein from the gene following expansion of the number of CTG trinucleotide repeats, leads to the disease phenotype. The molecular mechanisms leading the number of CTG repeats to increase from the range of 6 to 50 found in normal individuals to the greater than 50 repeats found in the chromosomes of diseased individuals are unknown. We have examined the consequences of CTG repeat expansion for nucleosome assembly and positioning. Short CTG repeats are found within the most favored DNA sequences yet defined for nucleosome assembly. We find that as few as six CTG repeats will facilitate nucleosome assembly to a similar extent as the fifty or more repeats found in disease genes. Thus an increase in nucleosome stability on expansion of existing triplet repeats is unlikely to explain the acquisition of the disease phenotype. However, the CTG repeat sequence is efficiently wrapped around the histone octamer, preferring to associate with histones at the nucleosomal dyad. Thus short segments CTG repeat sequence will facilitate the assembly of a stable positioned nucleosome which might contribute to the expansion phenomenon and the functional organization of chromatin.

Expansion and methylation of CGG repeat sequences is associated with Fragile X syndrome in humans. We have examined the consequences of CGG repeat expansion and methylation for nucleosome assembly and positioning on the Fragile X Mental Retardation gene 1 (FMR1) gene. Short unmethylated CGG repeats are not particularly favored in terms of affinity for the histone octamer or for positioning of the reconstituted nucleosome. However, upon methylation their affinity for the histone octamer increases and a highly positioned nucleosome assembles with the repeat sequences found adjacent to the nucleosomal dyad. Expansion of these CGG repeats abolishes the preferential nucleosome assembly due to methylation. Thus the expansion and methylation of these triplet repeats can alter the functional organization of chromatin, which may contribute to alterations in the expression of the FMR1 gene and the disease phenotype. Thus a common feature of triplet expansions in human disease genes in the assembly of a unusually stable nucleosome. Future studies will explore the functional consequences of this specialized chromatin structure.

The amino-terminal tails of the core histones and the translational position of the TATA box determine TBP/TFIIA association with nucleosomal DNA

We have established that the TATA binding protein (TBP) in the presence of TFIIA recognizes the TATA box in nucleosomal DNA dependent on the dissociation of the amino-terminal tails of the core histones from the nucleosome and the position of the TATA box within the nucleosome. We have examined TBP/TFIIA access to the TATA box with this sequence placed in four distinct rotational frames with reference to the histone surface, and at three distinct translational positions at the edge, side and dyad axis of the nucleosome. Under our experimental conditions, we find that the preferential translational position at which TBP/TFIIA can bind the TATA box is within linker DNA at the edge of the nucleosome, and that binding is facilitated if contacts made by the amino-terminal tails of the histones with nucleosomal DNA are eliminated. TBP/TFIIA binding to DNA at the edge of the nucleosome occurs with the TATA box in all four rotational positions. This is indicative of TBP/TFIIA association directing the dissociation of the TATA box from the surface of the histone octamer.

POST TRANSCRIPTIONAL CONTROL OF GENE EXPRESSION

Coupling transcription to RNA splicing

The project directed by Hui Ge concerns the coupling between transcription and RNA splicing. Using standard chromatography the transcriptional coactivator PC4 copurifies as a complex with a novel protein p75. Further analysis of proteins that interact with p75 reveal an additional association with ASF a splicing regulatory. ASF regulates alternative 5' splice site selection. Thus p75 has the potential to serve as a coupling factor between transcriptional regulatory mechanisms and the control of splicing.

Coupling transcription to translation

In earlier work we have established that translational fate in a Xenopus oocyte was coupled to the transcription process. A major contributor to this coupling is the mRNA packaging protein FRGY2. We have examined the determinants of the translational repression of mRNA by the Xenopus oocyte-specific Y-box protein FRGY2 using in vitro and in vivo assays. In vitro reconstitution of mRNP complexes demonstrates that the sequence-specific RNA-binding Cold Shock Domain is not required for translational repression, whereas the RNA-binding C-terminal Tail domain is essential. However microinjection of reconstituted mRNPs into Xenopus oocytes demonstrates that although translational repression occurs in the absence of consensus RNA binding sequences for FRGY2, the presence of FRGY2 recognition elements within mRNA potentiates translational repression. Analysis of the in vivo assembly of mRNP shows that the

Cold Shock Domain alone is not stably incorporated into mRNP, whereas the C-terminal Tail domain is sufficient for stable incorporation. We suggest that translational repression of mRNA by FRGY2 is favored by sequence selective recognition of RNA sequences by the Cold Shock Domain. However translational repression in vitro and the assembly of mRNP in vivo requires the relatively non-specific interaction of the C-terminal Tail domain with mRNA. Thus two distinct domains of FRGY2 are likely to contribute to translational control.

ANALYSIS OF THE S-PHASE CHECKPOINT IN HIGHER EUKARYOTES

The Unit on Cell Cycle Regulation directed by Mary Dasso seeks to discover the mechanism by which cells transmit and interpret signals that coordinate nuclear activities with each other and with the cell cycle. In particular, a focus is on understanding the control of cell division with respect to the completion of DNA replication, since mitosis is inhibited by signals from the nucleus until DNA synthesis is complete. Two molecular approaches are pursued: 1) the Ran GTPase pathway is investigated, which is required for nuclear transport and for coordination of nuclear activities to the cell cycle. 2) Cellular mRNAs are characterized whose stability is regulated by the S phase checkpoint, and those protein products may be required for the initiation of mitosis.

Ran-associated proteins in Xenopus extracts

Mutants that interfere with Ran or RCC1, Ran's guanine nucleotide exchange factor, have a broad spectrum of defects in vivo that include abnormalities in nuclear transport, cell cycle progression, nuclear structure and RNA metabolism. It has been unambiguously demonstrated that Ran is required for nuclear protein import in a permeabilized cell assay, but it is currently unresolved whether the pleiotropic defects observed in these mutants exclusively reflect nuclear transport deficiencies or whether Ran may have multiple functions in the cell. In either case, an understanding of the Ran GTPase pathway will be essential for understanding the fundamental cell biology of the nucleus.

In order to gain a better understanding of the Ran GTPase pathway, Xenopus egg extracts have been used for the purification and characterization of pathway components. Bacterially expressed glutathione S transferase-Ran fusion protein (GST-Ran) is a useful reagent for rapid and simple affinity purification procedures. GST-Ran associates specifically with at least ten extract proteins. These Ran interacting proteins (Rips) include: RanBP2/Nup358, Nup153, Importin β, hsc70, RCC1, and RanBP1. RanBP2 and Nup153 are nuclear pore proteins, and Importin β is required for targeting transport substrates to the nuclear pore. On the basis of peptide sequence, a seventh Rip (p88) appears to be similar but not identical to Fug1/RanGAP1, the GTPase activating protein (GAP) for Ran. The specificity of this method is supported by the fact that a number of the Rips have previously been found to interact with Ran in other assays (RanBP1, RCC1, Importin β, RanBP2), or to be consistuents of the nuclear pore or to have roles in nuclear transport (hsc70, Nup153). The behavior of endogenous Ran and Rips in egg extracts was examined, in order to learn how these proteins behave in the context of a complex mixture of proteins resembling that found in the intact embryo. Data suggest that Ran associates tightly with Importin β in the presence of non-hydrolyzable GTP analogs, and that both proteins show increased association to RanBP2 under these conditions. RanBP2 is constitutively associated with p88, suggesting a possible model wherein nucleotide hydrolysis by Ran in the context of a Ran-Importin β-RanBP2-p88 complex may facilitate protein import.

RanBP1 and RCC1 act antagonistically in nuclear assembly and in nuclear transport

RanBP1 was among the first proteins found to interact with Ran through screening of a mouse cDNA expression library for proteins that bind to Ran-GTP. Like Ran, RanBP1 is ubiquitously expressed and highly conserved between species. RanBP1 is encoded by an essential gene in yeast, and it has been found to function as a guanine nucleotide dissociation inhibitor (GDI) for Ran-GTP *in vitro*. However, it remains mechanistically unclear how RanBP1 promotes nuclear transport because it is not required *in vitro* for protein import permeabilized tissue culture cell assays. In order to examine RanBP1's role in nuclear assembly, transport and DNA replication, a *Xenopus* RanBP1 homologue was cloned and used to generate recombinant RanBP1 protein and anti-RanBP1 antibodies. RanBP1 was removed from egg extracts by serial immunodepletions using affinity purified antibodies coupled to protein A Sepharose beads. Surprisingly, immunodepletion of RanBP1 from *Xenopus* egg extracts resulted in co-depletion of RCC1, suggesting that these proteins can form a stable complex in extracts. These extracts were examined for their ability to sustain nuclear assembly, DNA replication and nuclear transport. Extracts lacking both RanBP1 and RCC1 (co-depleted extracts) did not exhibit defects in any of these assays. Nuclei from co-depleted extracts also responded normally to the addition of recombinant cyclin B protein, undergoing nuclear envelop breakdown and chromatin condensation that was indistinguishable from that seen in control reactions. Addition of either recombinant RanBP1 or RCC1 to co-depleted interphase extracts caused abnormal nuclear assembly, inhibited nuclear transport and blocked DNA replication in a manner that could be rescued by further addition of RCC1 or RanBP1, respectively. These results suggest that neither RanBP1 or RCC1 is essential for interphase nuclear functions in the absence of the other protein. The results further suggest that the balance of RCC1 and RanBP1 is critical for proper nuclear assembly and function *in vitro*.

The regulation of mRNA stability in the cell cycle

Despite the importance of the DNA replication checkpoint for maintaining genetic stability in eukaryotic cells, many of the intermediary components in the checkpoint pathway coupling the onset of mitosis to the completion of DNA replication remain to be identified. One mechanism by which mitotic onset is controlled is through changes in mRNA stability. In BHK cells, at least some of the mRNAs required for mitosis are unstable as long as DNA replication is actively occurring. These mRNAs become stable if DNA replication is blocked by drugs that inhibit DNA replication (e.g., hydroxyurea). Under these conditions, the cells are competent to enter mitosis in response to the addition of drugs such as caffeine. When the replication inhibitors are removed and DNA replication resumes, the mRNAs are rapidly destabilized and the cells lose their competence to enter mitosis in response to caffeine. These properties have allowed the identification of regulated mRNAs by the technique of differential display.

To date, approximately half of the mRNAs present in interphase BHK cells have been screened by differential display for their patterns of stability. In this screen, 23 mRNAs have been found whose display patterns conform to that expected from messages whose stability is controlled by ongoing replication. Of these, six have been screened by Northern blot analysis. Those mRNAs whose abundance does not allow screening by Northern blot analysis will be screened using an RNase protection assay. Each of the mRNAs that give appropriate signals in the Northern blot analysis will be examined to determine their half-lives in the presence or absence of ongoing replication.

MITOTIC CHROMOSOME CONDENSATION AND SEGREGATION

The structure and function of eukaryotic chromosomes are understood relatively well at the 'macro' level, i.e. chromosomes exist in two major forms: decondensed and condensed, performing respectively two major functions: template-based synthesis of nucleic acids and accurate distribution between the daughter cells. Extensive studies have been done at the molecular level to solve the fine structure and positioning of nucleosomes. However, there is an obvious gap in data concerning structure and function of chromatin as a sophisticated nucleoprotein complex undergoing remarkable changes during the cell cycle.

Alexander Strunnikov studies the properties of a new family of chromosomal components: the SMC-family of proteins that regulate chromosome structure and maintenance. The SMC-family is evolutionary conserved and at present is subdivided into at least three subfamilies based on sequence comparison. In budding yeast these subfamilies are represented by two essential genes SMC1 and SMC2 and a newly discovered gene SMC3. Representatives of this family of proteins are found now in other fungi, bacteria, nematodes, and several vertebrates where they are shown to be structural elements of chromosome organization. Cytological and biochemical properties of Smc1p and Smc2p suggest that these proteins are involved in the formation of the higher order chromosome structure. Studies are under way to determine the enzymatic activity, structural organization and regulation of these proteins. This approach includes, first of all, biochemical purification of the whole set of interacting proteins from the native system (yeast) as well as the homogeneously pure recombinant protein from the heterologous system (baculovirus-infected insect cells). Corresponding purified and semipurified proteins are directly tested for their role in chromosome condensation using as a substrate the eukaryotic chromatin reconstructed in vitro either from purified histones or from the nuclear extracts. All mutants in the genes for putative chromosomal proteins undergo extensive screening for genetically interacting loci. High-copy supressors of smc mutations have been isolated already. All three are previously uncharacterized genes and their physical interaction with SMC-proteins is being tested. In one case direct interaction is documented between the Smc1 protein and the product of an essential and evolutionary conserved suppressor gene. The mammalian homologues of the yeast proteins are subjected to the screen for protein-protein interactions in vivo (two-hybrid selection) and the mouse homologues of the yeast proteins are being cloned via a PCR-strategy in order to isolate the corresponding genomic DNA fragments. These genomic clones to be used for genetic studies of the corresponding genes in the mammalian cells (disruption and homozygosis of the essential genes and studies of the various mutations introduced into ectopically expressed cDNA).

GENE REGULATION BY THYROID HORMONE

Transcriptional regulation and chromatin disruption by thyroid hormone receptors

Yun-Bo Shi directs research as to how thyroid hormone regulates gene expression by controlling the transcription of target genes directly through thyroid hormone receptors (TRs). Work from other laboratories on TRs in birds and mammals has shown that TRs belong to the superfamily of the steroid hormone receptors. These receptors are sequence specific DNA binding proteins that regulate the transcription of genes containing their binding sites (TREs or thyroid hormone response elements for TRs) in a ligand dependent manner. While TRs can bind to TREs as monomers and homodimers, they most likely function as heterodimers formed with RXRs (9-cis retinoic acid receptor).

TH is the causative agent of amphibian metamorphosis. Toward investigating the roles of TR/RXR during this complex transformation, we have previous determined the mRNA levels of TR (TRα and TRβ) and RXR (RXRα and RXRγ) genes during Xenopus laevis metamorphosis in different organs. In general, TR and RXR genes are coordinately regulated in all organs and their expression correlates with organ specific transformation. Furthermore, we have reconstituted a thyroid hormone-dependent transcription system in Xenopus oocytes to study the function of Xenopus TR/RXR heterodimers. We have reconstituted in vivo the TH-regulated TRβA promoter in chromatin and studied the effect TR/RXR on the promoter activity. When a double stranded (ds) promoter plasmid is injected into oocytes, it is chromatinized such that the promoter is highly active. In contrast, when a single stranded (ss) promoter plasmid is injected into oocytes, it is rapidly replicated and undergoes replication-coupled chromatin assembly to produce a template of a very low basal promoter activity. Our earlier work has found that heterodimers of TR and RXR within the chromatin templates, generated by injection of either ds or ss DNA containing the TRβA promoter, can alteratively repress or active transcription dependent upon the absence or presence of TH. In addition, liganded TR/RXR heterodimers cause strong chromatin disruption and transcriptional activation while the unliganded receptors can bind to the TRE in chromatin without any detectible effect on the chromatin structure. We have now demonstrated that this chromatin disruption by liganded TRs is not restricted to the receptor binding site (TRE), instead it extends over a region encompassing at least several contiguous nucleosomes. Analysis of the supercoiling densities of the promoter plasmid before and after the addition of TH has revealed that TH-bound TR/RXR heterodimers cause chromatin disruption equivalent to a loss of 5 nucleosomes, which is about 20% of those present on the minichromosome (4500bp of DNA). This drastic chromatin remodeling activity of the TR is intimately associated with transcriptional activation by the receptor. Thus, we have identified a region of TRs within the C-terminal transcriptional activation domain that is necessary for both chromatin disruption and transcriptional activation but not for DNA or hormone binding. On the other hand, the chromatin disruption is separable from actual transcription process itself. This is demonstrated first by using α-amanitin, which can block transcription but not TH-induced, TR-mediated chromatin disruption. In addition, promoter deletion constructs that fail to activate transcription but bear the TRE can nonetheless mediate the same chromatin disruption in response to the binding by liganded TRs. However, the presence of the TRE is essential for such chromatin remodeling. These results demonstrate that chromatin disruption is an active process mediated by TRE-bound, liganded TRs and can occur regardless of the actual state of the transcription process. It remains to be tested whether this chromatin remodeling is necessary and/or sufficient for transcriptional activation by TRs.

Identification and characterization of a mutant TR in Xenopus laevis

While investigating the transcriptional properties of Xenopus TRs in the above mentioned oocyte transcriptional system, we discovered that the previously cloned Xenopus TRαB functioned poorly as an activator. Futhermore, the TRαB-RXR heterodimer has a drastically reduced affinity for a TRE. Site-directed mutagenesis shows that two of the seven amino acid residues that differ between TRαA and TRαB are responsible for the defect in TRαB function. These two residues are in the D domain, which is located between the DNA and hormone binding domains, and affect the DNA binding by both TR/RXR heterodimers and TR homodimers. In contrast, heterodimer formation with RXRs is not affected as demonstrated by coimmunoprecipitation and dominant-transcriptional inhibition experiments. Analysis of the expression of TRαA and TRαB indicates that both are expressed at similar levels in metamorphosing tadpoles. However, by recloning TRαB from tadpoles, we have demonstrated that the residues, which affect TRαB function when mutated, are identical between the new TRαB clones and TRαA. Thus, our experiments have discovered the first amphibian TR mutant. This mutant TR (the TRαB reported originally by Yaoita et al., 1990, PNAS, 87, 7090-7094) has two mutations in the D domain between the DNA and hormone binding domains. Both mutations involve a neutral residue to aspartic acid substitution. Based on a recent crystal structure of a complex between a TRE and a TR-RXR heterodimer, these two residues make either direct or indirect, via a water molecule, hydrogen-bonds with the phosphates of the TRE. Thus, the charge-charge repulsion between the phosphate backbone of the TRE and the mutated residues may be responsible for functional defects in the mutant.

Cell-cell and cell-extracellular matrix interactions during tissue remodeling

Each metamorphosing organ consists of many different cell types. Some of them are in direct contact with each other. Others are separated by extracellular matrices (ECM). Proper interactions among these cells are likely to be important for tissue remodeling. Intestinal remodeling serves as an ideal system to study cell-cell and cell-ECM interactions. The larval intestine consists of mostly a single layer of epithelial cell organized in a tubular structure with little connective tissue or muscles.

As the endogenous TH concentration rises, the connective tissue starts to increase in thickness and larval epithelial cells undergo apoptosis (programmed cell death). Concurrently, adult epithelial cells, whose origin is yet unknown, proliferate as cell islets. Around the time when cell death is mostly complete, adult epithelial cell differentiation is initiated. Within a few days, a multiple folded adult epithelium is developed, which is surrounded by elaborate connective tissue and muscle.

Among the TH-response genes that we have isolated during Xenopus intestinal remodeling are those encoding matrix metalloproteinases and a putative morphogen, hedgehog. Our studies have suggested that these genes are involved in regulation cell-cell and cell-ECM interactions during metamorphosis.

Xenopus sonic hedgehog as a potential morphogen

The hedgehog gene was first cloned as a segment polarity gene in Drosophila. Its homologs in mammals and birds are capable of functioning as morphogens in processes such as neural induction and limb morphogenesis. We have found that the Xenopus sonic hedgehog gene is activated by TH.

Interestingly, in situ hybridization and immunohistochemical analyses have demonstrated that the hedgehog gene is expressed in only the epithelial cells. However, cells of the pre- and post-metamorphic intestine have little hedgehog expression. The highest levels of hedgehog are found in what appear to be the proliferating islet cells. These results suggest that hedgehog may function to stimulate epithelial cell proliferation and/or initiate adult epithelial cell differentiation and morphogenesis. This role of hedgehog could be either through an autocrine pathway or by first affecting the connective tissue, which in turn influences epithelial development.

Matrix metalloproteinases (MMPs) in tissue remodeling

Cell-cell interactions can also be regulated through the modification ECM. Furthermore, ECM can directly affect the cells they contact. ECM remodeling is mediated in a large part by MMPs, each of which can degrade different but often overlapping components of ECM. We have previously reported the characterization of Xenopus stromelysin-3 (ST-3) gene, which encode a putative matrix metalloproteinase (MMP). Its mRNA is present only in the fibroblastic cells of the remodeling intestine. We have found by in situ hybridization that ST-3 is highly expressed in the fibroblastic cells surrounding the epithelium at the time when basal lamina, which separates the epithelium and the connective tissue, is remodeled to a thick, multiple folded, but more permeable structure that allows extensive cell-cell contacts between the fibroblasts and developing adult epithelium, and crossing of macrophages into the degenerating epithelium. While the role of ST-3 in the ECM modifications remains to be established, such ECM changes may affect cell behavior, e.g., larval epithelial cell death and adult epithelial proliferation and differentiation.

In contrast to ST3, a few other MMP genes have very different expression profiles. In particular, using a human cDNA probe for gelatinase A, we have found that the putative frog gelatin A is activated in the intestine later than ST3, i.e., when larval epithelial cell death is essentially complete. More recently, we have cloned a novel collagenase gene (Col4) from metamorphosing intestine. This new MMP gene is expressed throughout intestinal development but its mRNA is a few fold higher at same time when the gelatinase A gene is highly expressed, suggesting that like gelatinase A, Col4 participates in ECM removal after cell death and/or ECM remodeling during adult epithelial morphogenesis. This contrasts the proposed role for ST3. The earlier expression of ST3 implicates a role in initiating larval epithelial apoptosis and adult epithelium development.

Toward investigation the function of MMPs, we have over-expressed the new MMP, Col4, in E. coli. We have demonstrated that the Xenopus Col4 can degrade type I collagen and gelatine with similar characteristics as human Col1. However, unlike human Col1, the Xenopus Col4 can not digest casein. Further studies are needed to prove its role during frog metamorphosis.

Regulation of epithelial cell fate by TH in vitro

To investigate the mechanism underlying the TH-induced intestinal epithelial apoptosis, we have initiated an in vitro study. Premetamorphic tadpole intestine is digested to isolate larval epithelial cells and fibroblasts. These cells are then cultured on plastic dishes in vitro in the presence or absence of TH. In support of the in vivo observations, epithelial cells respond to TH by undergoing cell death while fibroblasts survive the TH treatment. The TH-induced cell death has been shown to be apoptotic by several assays. These include cell cycle analysis by FACS, an ELISA assay for DNA fragmentation, and direct analysis of DNA fragmentation by agarose gel electrophoresis. Future studies will be directed toward characterization the factors involved in this apoptosis.

LABORATORY OF MOLECULAR EMBRYOLOGY
Chief. Alan P. Wolffe, Ph.D.

Tenure track investigators
Mary Dasso, Ph.D.
Yun-Bo Shi, Ph.D.
Alexander Strunnikov, Ph.D.

Senior Fellows
Hui Ge, Ph.D.

Post-doctoral Fellows
Sashko Damjanovski
Cathy Jozwik
Nicolletta Landsberger
Qiao Li
Robert Pu
Monika Puzianowska-Kuznicka
Takashi Sera
Hisato Saitoh
Yuan Su
Karan Wassarman

Guest researchers
Anne Gegonne
James Godde
Michael Herrler
Axel Imhof
Ken Matsumoto
Danielle Patterton
John Strouboulis
Jiemin Wong

PUBLICATION

Dasso M. The role of the Ran GTPase pathway in cell cycle control and interphase nuclear functions. Prog Cell Cycle Res 1995;1:163-172.

Dimitrov S, Wolffe AP. Remodeling somatic nuclei in *Xenopus laevis* egg extracts: molecular mechanisms for the selective release of histone H1 and H1° from chromatin and the acquisition of transcriptional competence. EMBO J 1996;15:(in press).

Freeman L, Kurumizaka H, Wolffe AP. Functional domains for assembly of histones H3 and H4 into the chromatin of Xenopus embryos. Proc Natl Acad Sci USA 1996;(in press).

Godde JS, Wolffe AP. Nucleosome assembly on CTG triplet repeats. J Biol Chem 1996;271:15222-15229.

Godde JS, Nakatani Y, Wolffe AP. The aminio-terminal tails of the core histones and the translational position of the TATA box determine TBP/TFIIA association with nucleosomal DNA. Nucl Acids Res 1995;23:4557-4564.

Godde JS, Kass SU, Hirst MC, Wolffe AP. Nucleosome assembly on methylated CGG triplet repeats in the Fragile X Mental Retardation Gene 1 promoter. J Biol Chem 1996;(in press).

Hayes JJ, Dimitrov S, Wolffe AP. Physical and chemical analysis of the dynamics of nucleosome and chromatin structure. Chemtracts 1995;5:269-290.

Hayes JJ, Kaplan R, Ura K, Pruss D, Wolffe AP. Basic residues within a putative DNA-binding surface in the globular domain of linker histones are not essential for specific binding to the nucleosome core. J Biol Chem 1996;(in press).

Ishizuya-Oka A, Ueda S, Shi Y-B. Transient expression of stromelysin-3 mRNA in the amphibian small intestine during metamorphosis. Cell Tissues Res 1996;283:325-329.

Kass SU, Wolffe AP. Histones, histone modifications and the inheritance of chromatin structure. Epigenetics Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1996;(in press).

Kiang JG, Shi Y-B. Commentary: The third annual joint scientific symposium of NIH/FDA CAA and Washington DC Chapter of SCBA: Advances in Biological Sciences. J Biomedical Science 1996;3:133.

Landsberger N, Wolffe AP. Control of gene expression by chromatin structure. Frontiers in Biology, Enciclopedia Italiana 1996;(in press).

Matsumoto K, Meric F, Wolffe AP. Translational repression dependent on the interaction of the Xenopus Y-box protein FRGY2 with mRNA: the role of the Cold Shock Domain, the Tail Domain and selective RNA sequence recognition. J Biol Chem 1996;(in press).

Nightingale K, Dimitrov S, Reeves R, Wolffe AP. Evidence for a shared structural role for HMG1 and linker histones B4 and H1 in organizing chromatin. EMBO J 1996;15:548-561.

Nightingale KP, Pruss D, Wolffe AP. A single high affinity binding site for histone H1 in a nucleosome containing the Xenopus borealis 5S ribosomal RNA gene. J Biol Chem 1996;271:7090-7094.

Patterton D, Wolffe AP. Developmental roles for chromatin and chromosomal structure. Dev Biol 1996;173:2-13.

Pruss D, Bartholomew B, Persinger J, Hayes J, Arents G, Moudrianakis EN, Wolffe AP. An asymmetric model for the nucleosome: a binding site for linker histones inside the DNA gyres. Science 1996;(in press).

Puzianowska-Kuznicka M, Shi Y-B. Nuclear factor I as a potential regulator during post-embryonic organ development. J Biol Chem 1996;271:6273-6282.

Reeves R, Wolffe AP. Substrate structure exerts a dominant constraint on binding of the nonhistone protein HMG1 (Y) to free-and nucleosomal-DNA. Biochemistry 1996;35:5063-5074.

Saitoh H, Cooke CA, Burgess WH, Earnshaw WC, Dasso M. Direct and indirect association of the small GTPase Ran with nuclear pore proteins and soluble transport factors: Studies in Xenopus laevis egg extracts. Mol Biol Cell 1996;(in press).

Shi Y-B. Thyroid hormone-regulated early and late genes during amphibian metamorphosis. In Metamorphosis: Post-embryonic reprogramming of gene expression in amphibian and insect cells. (Eds. LI Gilbert, JR Tata and BG Atkinson) pp. 505-538. Academic Press, New York.

Shi Y-B, Ishizuya-Oka A. Biphasic intestinal development in amphibians: Embryogenesis and remodeling during metamorphosis. Current Topics in Developmental Biology 1996;32:205-235.

Shi Y-B, Stolow MA, Puzianowska-Kuznicka M, Wong J. Les genes de la metamorphose. La Recherche (French) 1996;286:58-64.

Shi Y-B, Wong J, Puzianowska-Kuznicka M, Stolow MA. Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: Roles of thyroid hormone and its receptors. BioEssays 1996;18:391-399.

Shi Y-B, Wong J, Puzianowska-Kuznicka M. Thyroid hormone receptors: Mechanisms of transcriptional regulation and roles during frog development. J Biomedical Science (in press).

Strouboulis J, Wolffe, AP. The functional compartmentalization of the nucleus. J Cell Sci 1996;(in press).

Ura K. In vitro studies of chromatin structure and transcriptional regulation. Protein, Nucleic Acid and Enzyme 1996;9:1385-1390.

Ura K, Wolffe AP. Reconstruction of transcriptionally active and silent chromatin. Methods Enzymol 1996;274:257-271.

Ura K, Nightingale K, Wolffe AP. Differential association of HMG1 and linker histones B4 and H1 with dinucleosomal DNA: structural transitions and transcriptional repression. EMBO J 1996;15:(in press).

Wolffe AP. The cold stress response in bacteria. Science Progress 1995;78:301-310.

Wolffe AP. Histone deacetylase: a regulator of transcription. Science 1996;272:371-372.

Wolffe AP. Chromatin structure and DNA replication: implications for transcriptional activity. DNA replication in eukaryotic cells. Cold Spring Harbor Press, CSH 1996;pp. 271-293.

Wolffe AP. Histone modification and transcriptional competence. Nuclear organization, chromatin structure and gene expression. Oxford University Press, Oxford 1996;(in press).

Wolffe AP, Meric F. Coupling transcription to translation: a novel site for the regulation of eukaryotic gene expression. International Journal of Biochemistry and Cell Biology 1995;28:247-257.

Wolffe AP, Pruss D. Deviant nucleosomes: the functional specialization of chromatin. Trends in Genetics 1996;12:58-62.

Wolffe AP, Pruss D. Hanging on to histones. Curr Biol 1996;6:234-237.

Wolffe AP, Pruss D. Targeting chromatin disruption: transcription factors that acetylate histones. Cell 1996;84:817-819.

Wong J, Shi Y-B, Wolffe AP. A role for nucleosome assembly in both silencing and activation of the Xenopus TRβA gene by the thyroid hormone receptor. Genes and Develop 1995;9:2696-2711.

Project Numbers - Annual Report 1996

LABORATORY OF MOLECULAR EMBRYOLOGY (LME)

Z01 HD 01900-06	Developmental Regulation of Differential Gene Expression Alan Wolffe, M.D.
Z01 HD 01901-02	Gene Regulation by Thyroid Hormone during Tissue Remodeling Yun-Bo Shi, Ph.D.
Z01 HD 01902-02	Analysis of the S Phase Checkpoint in Higher Eukaryotes Mary Dasso, Ph.D.
Z01 HD 01903-01	Role of SMC-proteins and Other Chromosomal Proteins in Mitotic Chromosome Segregation Alexander Strunnikov, Ph.D.

Z01 HD 01904-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01900-06 LME

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Developmental Regulation of Differential Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Alan Wolffe, Visiting Scientist LME/NICHD

OTHERS: Hui Ge, Senior Staff Fellow LME/NICHD
Anne Gegonne, Adjunct Scientist LME/NICHD
James Godde, Adjunct Scientist LME/NICHD
Michael Herrler, Adjunct Scientist LME/NICHD
Axel Imhof, Adjunct Scientist LME/NICHD
Cathy Jozwik, IRTA Fellow LME/NICHD
Nicoletta Landsberger, Visiting Fellow LME/NICHD
Qiao Li, Visiting Fellow LME/NICHD
Ken Matsumoto, Adjunct Scientist LME/NICHD
Danielle Patterton, Adjunct Technician LME/NICHD
Takashi Sera, Visiting Fellow LME/NICHD
John Strouboulis, Adjunct Scientist LME/NICHD
Karen Wassarman, IRTA Fellow LME/NICHD

COOPERATING UNITS *(if any)*

Laboratory of Molecular Embryology, NICHD (Y.-B. Shi); Université de Lausanne (W. Wahli); University of Nijmegen (R.N.H. Konings); Friedrich Miescher Laboratorium der Max Planck-Gesellschaft (R. Rupp); Southern Illinois University at Carbondale (B. Bartholomew); The Johns Hopkins University (E.N. Moudrianakis)

LAB/BRANCH

Laboratory of Molecular Embryology

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-2710

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
15	15	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our work is focussed on the molecular mechanisms responsible for establishing and maintaining stable states of gene expression during vertebrate embryogeneis. Progress has been achieved in the following key areas:

1. We have probed the internal architecture of a positioned nucleosome with photoactivatable cross-linking reagents to determine the placement of histones along the DNA molecule. We propose a new model of the nucleosome in which the winged-helix domain of the linker histone is asymmetrically located inside the gyres of DNA that also wrap around the core histones.

2. We have determined that HMG 1, linker histone B4 and H1 share a structural role in chromatin, yet have distinct functional consequences for the transcription of chromatin templates. These results explain the transcriptional promiscuity of early embryonic chromatin.

3. We have discovered that triplet repeat expansions associated with human disease genes including Fragile X Mental Retardation Gene 1 and the Dentatorubral-pallidoluysian Atrophy Gene share the common property of assembling nucleosomes of extraordinary stability. This stability might make a major contribution to the expansion of these repeats.

4. We have found the molecular chaperone nucleoplasmin to have a key role in remodeling somatic nuclei during their acquisition of totipotency in *Xenopus* eggs.

5. We have determined that a role for nucleosome assembly exists in both the silencing and activation of the *Xenopus* TRβA gene by the thyroid hormone receptor.

Objectives:

The object of this project is to determine the molecular processes responsible for establishing and maintaining stable states of gene activity during vertebrate development.

Methods Employed:

High resolution gel electrophoresis is used to resolve DNA sequence at the nucleotide level, various chemical and enzymatic probes determine the nature and pattern of transcription factor binding to DNA. Transcription factors and RNA polymerase are fractionated and purified by standard chromatographic procedures. High efficiency in vitro transcription assays are used to assess the function of transcription factor binding to specific DNA sequences and the function of transcription factor interactions with other transcription factors. These interactions are quantitated further by competitive footprinting and gel retardation assays. Chromatin structure of active and inactive genes is assessed using enzymatic and chemical probes, followed by gel electrophoresis and sucrose gradient sedimentation. Histones and transcription factors associated with chromatin are monitored by electrophoresis and immunoblotting using polyclonal antibodies. Sub-cellular localization is assessed using immofluorescence and microscopy. Chromatin is assembled in vitro using cell-free preparations of Xenopus eggs and embryos. Microinjection of oocytes and embryos is used to assess the functional role of DNA sequence and protein-nucleic acid interactions in vivo. mRNA is isolated, cDNA synthesized and fusion proteins expressed in bacteria. Mutant proteins are constructed by reverse genetic techniques and their functions assayed in vitro as described above.

Major Findings:

GENE REGULATION DURING EARLY EMBRYOGENESIS

A role for nucleosome assembly in both silencing and activation of the Xenopus TRβA gene by the thyroid hormone receptor

In collaboration with Dr. Y.-B. Shi (Unit on Molecular Morphogenesis, LME) we have assembled the thyroid hormone-inducible promoter of the Xenopus thyroid hormone receptor (TR)βA gene into chromatin using replication-coupled and -independent assembly pathways in vivo. We establish that heterodimers of TR and 9-cis retinoic acid receptors (RXR) can bind to their recognition sites within chromatin both in vivo and in vitro and alternately repress or activate transcription dependent on the absence or presence of thyroid hormone. Maximal transcriptional repression requires the presence of unliganded TR/RXR heterodimers during replication-coupled chromatin assembly. We demonstrate an increase in transcription directed by the TRβA promoter of over twow orders of magnitude in vivo, following the addition of thyroid hormone. This increase in transcription involves the relief of the repressed state that is established by the unliganded TR/RXR heterodimer during replication-coupled chromatin assembly. The association of thyroid hormone with the chromatin-bound TR/RXR heterodimer leads to the disruption of local chromatin structure in a transcription-independent process. Thus, chromatin structure has multiple roles in the regulation of TRβA gene expression in vivo: The

TR/RXR heterodimer recognizes the response element within chromatin, TR/RXR makes use of the chromatin assembly process to silence transcription more efficiently, and TR/RXR directs the disruption of local chromatin structure in response to thyroid hormone.

Remodeling of somatic nuclei in *Xenopus* egg extracts leading to the acquisition of transcriptional competence

We have examined the molecular mechanisms responsible for the remodeling of entire somatic erythrocyte nuclei in Xenopus laevis egg cytoplasm. These transitions in chromosomal composition are associated with the capacity to activate new patterns of gene expression and the reacquisition of replication competence. Somatic linker histone variants, H1, H1° and H5 are rapidly released from chromatin in egg cytoplasm, whereas the oocyte specific linker histone B4 and HMG1 are efficiently incorporated into remodeled chromatin. Core histones H2A and H4 in the somatic nucleus are phosphorylated during this remodeling process. These transitions recapitulate the chromosomal environment found within the nuclei of the early Xenopus embryo. We determine that phosphorylation of somatic linker histone variants is not essential for their release from chromatin, nor is competition with cytoplasmic stores of linker histone B4 and HMG1 essential for their release. We find that the molecular chaperone nucleoplasmin does have an essential role in the selective removal of linker histones from somatic nuclei. For Xenopus erythrocyte nuclei, this disruption of chromatin structure leads to the transcriptional activation of oocyte-specific genes. Our results provide a molecular explanation for the remodeling of chromatin in Xenopus egg cytoplasm, and indicate the capacity of molecular chaperones to independently disrupt a natural chromosomal environment thereby facilitating the transcription process.

CHROMATIN STRUCTURE AND FUNCTION

A new model for the nucleosome

Histone-DNA contacts within a nucleosome influence the function of trans-acting factors and the molecular machines required to activate the transcription process. We have probed the internal architecture of a positioned nucleosome using photoactivatable cross-linking reagents to determine the placement of histones along the DNA molecule. We find that the winged-helix domain of the linker histone is located inside the gyres of DNA that also wrap around the core histones. The preferential association of the linker histone to one side of the nucleosome generates an asymmetric particle that may impart a directionality to the folding of the chromatin fiber. This might be propagated by a polar head-to-tail arrangement of linker histone molecules along the nucleosomal array or chromatin fiber. The proposed orientation of the winged helix domain is also likely to favor interaction of the extended basic C-terminal tail of the linker histone with the linker DNA. Charge neutralization of the phosphodiester backbone of linker DNA by the C-terminal tail has a major role in chromatin compaction. Moreover the removal of the linker histone away from the dyad axis of the nucleosome would eliminate the need for the DNA at the entry and the exit of the nucleosome to come into close proximity. This also eliminates a central requirement of certain models of the chromatin fiber that have the linker histone uniquely localized at the center of the fiber.

Our model of the nucleosome provides a simple explanation for the influence of linker histones on transcription and nucleosome positioning. Histone H1 and H5 have been shown to direct the exact DNA sequences wrapped by the histone octamer. These linker histones also restrict nucleosome mobility. Since linker histones resemble other winged helix proteins, interactions with the major groove of DNA as suggested here would confer sequence selective recognition. Transcription factors of the winged helix class such as HNF3 have already been shown to position nucleosomes. Removal of histone H1 as seen on transcriptional activation of inducible promoters would remove a major positional signal for chromatin organization, and the resulting mobility of histone octamers with respect to DNA sequence could greatly facilitate transcriptional activation.

Differential association of HMG1 and linker histones B4 and H1 with nucleosomal DNA: structural transitions and transcriptional repression

The high mobility group proteins 1 and 2 (HMG1/2) and histone B4 are major components of chromatin within the nuclei assembled during the incubation of Xenopus sperm chromatin in Xenopus egg extract. To investigate their potential structural and functional roles, we have cloned and expressed Xenopus HMG1 and histone B4. Purified histone B4 and HMG1 form stable complexes with nucleosomes including Xenopus 5S DNA. Both proteins associate with linker DNA and stabilize it against digestion with micrococcal nuclease, in a similar manner to histone H1. However, neither histone B4 nor HMG1 influence the DNase I or hydroxyl radical digestion of DNA within the nucleosome core. HMG1/2 and histone B4 have a shared structural role in organizing linker DNA in the nucleosome.

We have examined further the structural and functional consequences of incorporating either histone H1, histone B4 or HMG1 into a synthetic dinucleosome containing two 5S rRNA genes. We found that all three proteins bind to linker DNA, stabilizing an additional 20 bp from micrococcal nuclease digestion and restrict nucleosome mobility. Histone H1 has the highest-affinity interaction with the dinucleosome; histone B4 and HMG1 associate with significantly reduced affinities. We found that histone H1 binds to the dinucleosome template with a dissociation constant (K_D) of 7.4 nM, whereas the K_D is 45 nM for histone B4 and 300 nM for HMG1. The K_Ds for the interaction of these proteins with naked DNA are 18 nM for H1, 80 nM for B4 and 300 nM for HMG1. The differences in association of these proteins with the dinucleosome are reflected in the efficiency with which the different proteins repress transcription from the 5S rRNA genes. Thus, although all three proteins can contribute to the organization of chromatin, the stability of the structures they assemble will vary. Our results provide a molecular explanation for the transcriptional promiscuity of Xenopus early embryonic chromatin, which is enriched in HMG1 and linker histone B4, but deficient in histone H1.

Nucleosome assembly on human disease genes

Expansions of CTG trinucleotide repeat sequences are now associated with several human genetic diseases. These include myotonic dystrophy, spinal and bulbar muscular atrophy, Huntington's disease, spinocerebellar ataxia, and dentatorubral-pallidoluysian atrophy. The CTG trinucleotide repeats are found associated with certain genes whose products are necessary for correct cell function. Aberrant gene expression or the synthesis of a mutant protein from the gene following expansion of the number of CTG trinucleotide repeats, leads to the disease phenotype. The molecular mechanisms leading the number of CTG repeats to increase from the range of 6 to 50 found in normal individuals to the greater than 50 repeats found in the chromosomes of diseased individuals are unknown. We have examined the consequences of CTG repeat expansion for nucleosome assembly and positioning. Short CTG repeats are found within the most favored DNA sequences yet defined for nucleosome assembly. We find that as few as six CTG repeats will facilitate nucleosome assembly to a similar extent as the fifty or more repeats found in disease genes. Thus an increase in nucleosome stability on expansion of existing triplet repeats is unlikely to explain the acquisition of the disease phenotype. However, the CTG repeat sequence is efficiently wrapped around the histone octamer, preferring to associate with histones at the nucleosomal dyad. Thus short segments CTG repeat sequence will facilitate the assembly of a stable positioned nucleosome which might contribute to the expansion phenomenon and the functional organization of chromatin.

Expansion and methylation of CGG repeat sequences is associated with Fragile X syndrome in humans. We have examined the consequences of CGG repeat expansion and methylation for nucleosome assembly and positioning on the Fragile X Mental Retardation gene 1 (FMR1) gene. Short unmethylated CGG repeats are not particularly favored in terms of affinity for the histone octamer or for positioning of the reconstituted nucleosome. However, upon methylation their affinity for the histone octamer increases and a highly positioned nucleosome assembles with the repeat sequences found adjacent to the nucleosomal dyad. Expansion of these CGG repeats abolishes the preferential nucleosome assembly due to methylation. Thus the expansion and methylation of these triplet repeats can alter the functional organization of chromatin, which may contribute to alterations in the expression of the FMR1 gene and the disease phenotype. Thus a common feature of triplet expansions in human disease genes in the assembly of a unusually stable nucleosome. Future studies will explore the functional consequences of this specialized chromatin structure.

The amino-terminal tails of the core histones and the translational position of the TATA box determine TBP/TFIIA association with nucleosomal DNA

We have established that the TATA binding protein (TBP) in the presence of TFIIA recognizes the TATA box in nucleosomal DNA dependent on the dissociation of the amino-terminal tails of the core histones from the nucleosome and the position of the TATA box within the nucleosome. We have examined TBP/TFIIA access to the TATA box with this sequence placed in four distinct rotational frames with reference to the histone surface, and at three distinct translational positions at the edge, side and dyad axis of the nucleosome. Under our experimental conditions, we find that the preferential translational position at which TBP/TFIIA can bind the TATA box is within linker DNA at the edge of the nucleosome, and that binding is facilitated if contacts made by the amino-terminal tails of the histones with nucleosomal DNA are eliminated. TBP/TFIIA binding to DNA at the edge of the nucleosome occurs with the TATA box in all four rotational positions. This is indicative of TBP/TFIIA association directing the dissociation of the TATA box from the surface of the histone octamer.

TRANSLATIONAL CONTROL

Translational repression dependent on the interaction of the Xenopus Y-box protein FRGY2 with mRNA

We have examined the determinants of the translational repression of mRNA by the Xenopus oocyte-specific Y-box protein FRGY2 using in vitro and in vivo assays. In vitro reconstitution of mRNP complexes demonstrates that the sequence-specific RNA-binding Cold Shock Domain is not required for translational repression, whereas the RNA-binding C-terminal Tail domain is essential. However microinjection of reconstituted mRNPs into Xenopus oocytes demonstrates that although translational repression occurs in the absence of consensus RNA binding sequences for FRGY2, the presence of FRGY2 recognition elements within mRNA potentiates translational repression. Analysis of the in vivo assembly of mRNP shows that the Cold Shock Domain alone is not stably incorporated into mRNP, whereas the C-terminal Tail domain is sufficient for stable incorporation. We suggest that translational repression of mRNA by FRGY2 is favored by sequence selective recognition of RNA sequences by the Cold Shock Domain. However translational repression in vitro and the assembly of mRNP in vivo requires the relatively non-specific interaction of the C-terminal Tail domain with mRNA. Thus two distinct domains of FRGY2 are likely to contribute to translational control.

Publications:

Dimitrov S, Wolffe AP. Remodeling somatic nuclei in Xenopus laevis egg extracts: molecular mechanisms for the selective release of histone H1 and H1° from chromatin and the acquisition of transcriptional competence. EMBO J 1996;15:(in press).

Freeman L, Kurumizaka H, Wolffe AP. Functional domains for assembly of histones H3 and H4 into the chromatin of Xenopus embryos. Proc Natl Acad Sci USA 1996;(in press).

Godde JS, Wolffe AP. Nucleosome assembly on CTG triplet repeats. J Biol Chem 1996;271:15222-15229.

Godde JS, Nakatani Y, Wolffe AP. The aminio-terminal tails of the core histones and the translational position of the TATA box determine TBP/TFIIA association with nucleosomal DNA. Nucl Acids Res 1995;23:4557-4564.

Godde JS, Kass SU, Hirst MC, Wolffe AP. Nucleosome assembly on methylated CGG triplet repeats in the Fragile X Mental Retardation Gene 1 promoter. J Biol Chem 1996;(in press).

Hayes JJ, Dimitrov S, Wolffe AP. Physical and chemical analysis of the dynamics of nucleosome and chromatin structure. Chemtracts 1995;5:269-290.

Hayes JJ, Kaplan R, Ura K, Pruss D, Wolffe AP. Basic residues within a putative DNA-binding surface in the globular domain of linker histones are not essential for specific binding to the nucleosome core. J Biol Chem 1996;(in press).

Kass SU, Wolffe AP. Histones, histone modifications and the inheritance of chromatin structure. Epigenetics Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1996;(in press).

Landsberger N, Wolffe AP. Control of gene expression by chromatin structure. Frontiers in Biology, Enciclopedia Italiana 1996;(in press).

Matsumoto K, Meric F, Wolffe AP. Translational repression dependent on the interaction of the Xenopus Y-box protein FRGY2 with mRNA: the role of the Cold Shock Domain, the Tail Domain and selective RNA sequence recognition. J Biol Chem 1996;(in press).

Nightingale K, Dimitrov S, Reeves R, Wolffe AP. Evidence for a shared structural role for HMG1 and linker histones B4 and H1 in organizing chromatin. EMBO J 1996;15:548-561.

Nightingale KP, Pruss D, Wolffe AP. A single high affinity binding site for histone H1 in a nucleosome containing the Xenopus borealis 5S ribosomal RNA gene. J Biol Chem 1996;271:7090-7094.

Patterton D, Wolffe AP. Developmental roles for chromatin and chromosomal structure. Dev Biol 1996;173:2-13.

Pruss D, Bartholomew B, Persinger J, Hayes J, Arents G, Moudrianakis EN, Wolffe AP. An asymmetric model for the nucleosome: a binding site for linker histones inside the DNA gyres. Science 1996;(in press).

Reeves R, Wolffe AP. Substrate structure exerts a dominant constraint on binding of the nonhistone protein HMG1 (Y) to free-and nucleosomal-DNA. Biochemistry 1996;35:5063-5074.

Strouboulis J, Wolffe, AP. The functional compartmentalization of the nucleus. J Cell Sci 1996;(in press).

Ura K. In vitro studies of chromatin structure and transcriptional regulation. Protein, Nucleic Acid and Enzyme 1996;9:1385-1390.

Ura K, Wolffe AP. Reconstruction of transcriptionally active and silent chromatin. Methods Enzymol 1996;274:257-271.

Ura K, Nightingale K, Wolffe AP. Differential association of HMG1 and linker histones B4 and H1 with dinucleosomal DNA: structural transitions and transcriptional repression. EMBO J 1996;15:(in press).

Wolffe AP. The cold stress response in bacteria. Science Progress 1995;78:301-310.

Wolffe AP. Histone deacetylase: a regulator of transcription. Science 1996;272:371-372.

Wolffe AP. Chromatin structure and DNA replication: implications for transcriptional activity. DNA replication in eukaryotic cells. Cold Spring Harbor Press, CSH 1996;pp. 271-293.

Wolffe AP. Histone modification and transcriptional competence. Nuclear organization, chromatin structure and gene expression. Oxford University Press, Oxford 1996;(in press).

Wolffe AP, Meric F. Coupling transcription to translation: a novel site for the regulation of eukaryotic gene expression. International Journal of Biochemistry and Cell Biology 1995;28:247-257.

Wolffe AP, Pruss D. Deviant nucleosomes: the functional specialization of chromatin. Trends in Genetics 1996;12:58-62.

Wolffe AP, Pruss D. Hanging on to histones. Curr Biol 1996;6:234-237.

Wolffe AP, Pruss D. Targeting chromatin disruption: transcription factors that acetylate histones. Cell 1996;84:817-819.

Wong J, Shi Y-B, Wolffe AP. A role for nucleosome assembly in both silencing and activation of the Xenopus TRβA gene by the thyroid hormone receptor. Genes and Develop 1995;9:2696-2711.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD01901-02 LME

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene Regulation by Thyroid Hormone during Tissue Remodeling

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Yun-Bo Shi, Senior Staff Fellow LME/NICHD

OTHERS:
Monika Puzianowska-Kuznicki, Visiting Fellow LME/NICHD
Melissa Stolow, Adjunct Scientist LME/NICHD
Jiemin Wong, Adjunct Scientist LME/NICHD
Yuan Su, Adjunct Scientist LME/NICHD
Sashko Damjanovski, Adjunct Scientist LME/NICHD

COOPERATING UNITS *(if any)*

Dept. of Anatomy, Dokkyo University, School of Medicine, Japan (A. Ishizuya-Oka); Section of Mol. Biol., LME, NICHD (A. Wolffe); Dept. of Chem. and Institute of Mol. Biophysics, Florida State Univ. (O. Sang)

LAB/BRANCH

Laboratory of Molecular Embryology

SECTION

Unit on Molecular Morphogenesis

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-5455

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.2	5.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The research in the Unit of Molecular Morphogenesis is focussed on the understanding of the molecular mechanism of amphibian metamorphosis. The control of this developmental process by thyroid hormone (TH) offers a unique paradigm in which to study genes that are important for post-embryonic organ development. We began to study metamorphosis by choosing the remodeling of the tadpole intestine in Xenopus laevis as a model system. The tadpole intestine is a simple tubular structure consisting of primarily a single layer of primary epithelial cells. During metamorphosis, it is transformed into a multiply folded adult epithelium with elaborate connective tussue and muscles through specific cell death and selective all proliferation and differentiation. We have many TH-response genes in the intestine during this transition. Among them are genes encoding matrix metalloproteinases (MMPs) and a putative morphogen, the Xenopus hedgehog. Thus, a major area of our work is to understand how these extracellular proteins influence organogenesis. Our evidence suggests that the Xenopus hedgehog functions in initiating and/or stimulating adult intestinal epithelial all proliferation and differentiation, in agreement with its morphogenic roles in other species. On the other hand, MMPs appear to be involved in ECM remodeling, which in turn influences cell behavior, especially larval epithelial apoptosis and adult cell proliferation. We have developed in vitro cell and organ culture systems which will allow us to further investigate how these proteins function to regulate cell-cell and cell-ECM interactions.

The second research area aims at investigating the function of thyroid hormone receptors (TRs) during metamorphosis. Our current work has demonstrated that TR/RXR (9-cis retinoic acid receptor) can function within a chromatin context and that transcriptional activation leads to chromatin disruption. We have revealed a number of interesting characteristic associated with TR function. In addition, we have identified and functionally characterized a mutant TR in Xenopus laevis.

Project Description:

Objectives:

To understand the molecular mechanism of gene regulation by thyroid hormone and the roles of thyroid hormone response genes, especially those influence cell-cell and cell-ECM interactions, during frog organ development.

Methods Employed:

In vitro biochemical studies of protein function and nucleic acid manipulation; functional investigation of transcription factors in oocytes and embryos; in situ hybridization and immunohistochemical analyses of gene expression; in vitro cell culture analysis of tadpole epithelial cell response to thyroid hormone

Major findings:

1. Thyroid hormone receptor function

(A) Transcriptional regulation and chromatin disruption by TH receptors (in collaboration with Dr. Alan Wolffe)

TH is known to regulate gene expression by controlling the transcription of target genes directly through thyroid hormone receptors (TRs). Work from other laboratories on TRs in birds and mammals has shown that TRs belong to the superfamily of the steroid hormone receptors. These receptors are sequence specific DNA binding proteins that regulate the transcription of genes containing their binding sites (TREs or thyroid hormone response elements for TRs) in a ligand dependent manner. While TRs can bind to TREs as monomers and homodimers, they most likely function as heterodimers formed with RXRs (9-cis retinoic acid receptor).

TH is the causative agent of amphibian metamorphosis. Toward investigating the roles of TR/RXR during this complex transformation, we have previous determined the mRNA levels of TR (TRα and TRβ) and RXR (RXRα and RXRγ) genes during Xenopus laevis metamorphosis in different organs. In general, TR and RXR genes are coordinately regulated in all organs and their expression correlates with organ specific transformation. Furthermore, we have reconstituted a thyroid hormone-dependent transcription system in Xenopus oocytes to study the function of Xenopus TR/RXR heterodimers. In collaboration with Dr. A. Wolffe, we have reconstituted in vivo the TH-regulated TRβA promoter in chromatin and studied the effect TR/RXR on the promoter activity. Dr. Wolffe's group has shown previously that when a double stranded (ds) promoter plasmid is injected into oocytes, it are chromatinized such that the promoter is highly active. In contrast, when a single stranded (ss) promoter plasmid is injected into oocytes, it is rapidly replicated and undergoes replication-coupled chromatin assembly to produce a template of a very low basal promoter activity. Our earlier work has found that heterodimers of TR and RXR within the chromatin templates, generated by injection of either ds or ss DNA containing the TRβA promoter, can alteratively repress or active transcription dependent upon the absence or presence of TH. In addition, liganded TR/RXR heterodimers cause strong chromatin disruption and transcriptional activation while the unliganded receptors can bind to the TRE in chromatin without any detectible effect on the chromatin structure. We have now demonstrated that this chromatin disruption by liganded TRs is not restricted to the receptor binding site (TRE), instead it extends over a region encompassing at least several contiguous nucleosomes. Analysis of the supercoiling densities of the promoter plasmid before and after the addition of TH has revealed that TH-bound TR/RXR heterodimers cause chromatin disruption equivalent to a loss of 5 nucleosomes, which is about 20% of those present on the minichromosome (4500bp of DNA). This drastic chromatin remodeling activity of the TR is intimately associated with transcriptional activation by the receptor. Thus, we have identified a region of TRs within the C-terminal transcriptional activation domain that is necessary for both chromatin disruption and transcriptional activation but not for DNA or hormone binding. On the other hand, the chromatin disruption is separable from actual transcription process itself. This is demonstrated first by using α-amanitin, which can block transcription but not TH-induced, TR-mediated chromatin disruption. In addition, promoter deletion constructs that fail to activate transcription but bear the TRE can nonetheless mediate the same chromatin disruption in response to the binding by liganded TRs. However, the presence of the TRE is essential for such chromatin remodeling. These results demonstrate that chromatin disruption is an active process mediated by TRE-bound, liganded TRs and can occur regardless of the actual state of the transcription process. It remains to be tested whether this chromatin remodeling is necessary and/or sufficient for transcriptional activation by TRs.

(B) Identification and characterization of a mutant TR in Xenopus laevis

While investigating the transcriptional properties of Xenopus TRs in the above mentioned oocyte transcriptional system, we discovered that the previously cloned Xenopus TRαB functioned poorly as an activator. Futhermore, the TRαB-RXR heterodimer has a drastically reduced affinity for a TRE. Site-directed mutagenesis shows that two of the seven amino acid residues that differ between TRαA and TRαB are responsible for the defect in TRαB function. These two residues are in the D domain, which is located between the DNA and hormone binding domains, and affect the DNA binding by both TR/RXR heterodimers and TR homodimers. In contrast, heterodimer formation with RXRs is not affected as demonstrated by coimmunoprecipitation and dominant-transcriptional inhibition experiments. Analysis of the expression of TRαA and TRαB indicates that both are expressed at similar levels in metamorphosing tadpoles. However, by recloning TRαB from tadpoles, we have demonstrated that the residues, which affect TRαB function when mutated, are identical between the new TRαB clones and TRαA. Thus, our experiments have discovered the first amphibian TR mutant. This mutant TR (the TRαB reported originally by Yaoita et al., 1990, PNAS, 87, 7090-7094) has two mutations in the D domain between the DNA and hormone binding domains. Both mutations involve a neutral residue to aspartic acid substitution. Based on a recent crystal structure of a complex between a TRE and a TR-RXR heterodimer, these two residues make either direct or indirect, via a water molecule, hydrogen-bonds with the phosphates of the TRE. Thus, the charge-charge repulsion between the phosphate backbone of the TRE and the mutated residues may be responsible for functional defects in the mutant.

2. Cell-cell and cell-extracellular matrix interactions during tissue remodeling

Each metamorphosing organ consists of many different cell types. Some of them are in direct contact with each other. Others are separated by extracellular matrices (ECM). Proper interactions among these cells are likely to be important for tissue remodeling. Intestinal remodeling serves as an ideal system to study cell-cell and cell-ECM interactions. The larval intestine consists of mostly a single layer of epithelial cell organized in a tubular structure with little connective tissue or muscles.

As the endogenous TH concentration rises, the connective tissue starts to increase in thickness and larval epithelial cells undergo apoptosis (programmed cell death). Concurrently, adult epithelial cells, whose origin is yet unknown, proliferate as cell islets. Around the time when cell death is mostly complete, adult epithelial cell differentiation is initiated. Within a few days, a multiple folded adult epithelium is developed, which is surrounded by elaborate connective tissue and muscle.

Among the TH-response genes that we have isolated during Xenopus intestinal remodeling are those encoding matrix metalloproteinases and a putative morphogen, hedgehog. Our studies have suggested that these genes are involved in regulation cell-cell and cell-ECM interactions during metamorphosis.

(A) Xenopus sonic hedgehog as a potential morphogen

The hedgehog gene was first cloned as a segment polarity gene in Drosophila. Its homologs in mammals and birds are capable of functioning as morphogens in processes such as neural induction and limb morphogenesis. We have found that the Xenopus sonic hedgehog gene is activated by TH.

Interestingly, in situ hybridization and immunohistochemical analyses have demonstrated that the hedgehog gene is expressed in only the epithelial cells. However, cells of the pre- and post-metamorphic intestine have little hedgehog expression. The highest levels of hedgehog are found in what appear to be the proliferating islet cells. These results suggest that hedgehog may function to stimulate epithelial cell proliferation and/or initiate adult epithelial cell differentiation and morphogenesis. This role of hedgehog could be either through an autocrine pathway or by first affecting the connective tissue, which in turn influences epithelial development.

(B) Matrix metalloproteinases (MMPs) in tissue remodeling (in collaboration with Drs. A. Ishizuya-Oka and Q. Sang)

Cell-cell interactions can also be regulated through the modification ECM. Furthermore, ECM can directly affect the cells they contact. ECM remodeling is mediated in a large part by MMPs, each of which can degrade different but often overlapping components of ECM. We have previously reported the characterization of Xenopus stromelysin-3 (ST-3) gene, which encode a putative matrix metalloproteinase (MMP). Its mRNA is present only in the fibroblastic cells of the remodeling intestine. In collaboration with Dr. Ishizuya-Oka, we have found by in situ hybridization that ST-3 is highly expressed in the fibroblastic cells surrounding the epithelium at the time when basal lamina, which separates the epithelium and the connective tissue, is remodeled to a thick, multiple folded, but more permeable structure that allows extensive cell-cell contacts between the fibroblasts and developing adult epithelium, and crossing of macrophages into the degenerating epithelium. While the role of ST-3 in the ECM modifications remains to be established, such ECM changes may affect cell behavior, e.g., larval epithelial cell death and adult epithelial proliferation and differentiation.

In contrast to ST3, a few other MMP genes have very different expression profiles. In particular, using a human cDNA probe for gelatinase A, we have found that the putative frog gelatin A is activated in the intestine later than ST3, i.e., when larval epithelial cell death is essentially complete. More recently, we have cloned a novel collagenase gene (Col4) from metamorphosing intestine. This new MMP gene is expressed throughout intestinal development but its mRNA is a few fold higher at same time when the gelatinase A gene is highly expressed, suggesting that like gelatinase A, Col4 participates in ECM removal after cell death and/or ECM remodeling during adult epithelial morphogenesis. This contrasts the proposed role for ST3. The earlier expression of ST3 implicates a role in initiating larval epithelial apoptosis and adult epithelium development.

Toward investigation the function of MMPs, we have over-expressed the new MMP, Col4, in E. coli. In collaboration with Dr. Q. Sang, we have demonstrated that the Xenopus Col4 can degrade type I collagen and gelatine with similar characteristics as human Col1. However, unlike human Col1, the Xenopus Col4 can not digest casein. Further studies are needed to prove its role during frog metamorphosis.

(C) Regulation of epithelial cell fate by TH in vitro

To investigate the mechanism underlying the TH-induced intestinal epithelial apoptosis, we have initiated an in vitro study. Premetamorphic tadpole intestine is digested to isolate larval epithelial cells and fibroblasts. These cells are then cultured on plastic dishes in vitro in the presence or absence of TH. In support of the in vivo observations, epithelial cells respond to TH by undergoing cell death while fibroblasts survive the TH treatment. The TH-induced cell death has been shown to be apoptotic by several assays. These include cell cycle analysis by FACS, an ELISA assay for DNA fragmentation, and direct analysis of DNA fragmentation by agarose gel electrophoresis. Future studies will be directed toward characterization the factors involved in this apoptosis.

Proposed course of projects:

Gene regulation by thyroid hormone receptors

Our studies so far have shown that TR/RXR heterodimer can regulate transcription from a chromatinized template, and disrupt chromatin upon transcriptional activation in the presence of TH. Furthermore, a C-terminal domain is required for both transcriptional activation and chromatin disruption. We would like to further investigate the mechanism of this disruption and to determine whether such chromatin remodeling is necessary and sufficient for transcriptional activation. Do TR and RXR interact with any factors to bring about chromatin structural changes? What are these factors? Are these also the same factors that are involved in promoter activation?

We plan to establish an in vitro transcription system to study TR/RXR function. We will make use of the TH-dependent TRβ promoter as a reporter and the in vitro transcription system that Dr. H. Ge (LME/NICHD) has developed.

In addition, we are also interested to study TR/RXR function in developing embryos. We will approach this by introducing TR/RXR into fertilized eggs and analyzing the expression of endogenous genes in these embryos with over-expressed TR/RXR or their mutants. Phenotypic analysis will also be carried out. Preliminary experiments have demonstrated that over-expression of wild type TR/RXR causes developmental defects and the addition of TH results in even more dramatic defects. Interestingly, all these defects show similarities to those observed in retinoic acid treated embryos. Further experiments will be direct to analyze the molecular bases.

Cell-cell and cell-ECM interactions

We plan to investigate further how hedgehog and MMPs participate in intestinal remodeling. We intend to analyze the temporal and spatial distribution of the proteins by Western blot and immunohistochemistry. To study the biological functions, we have now established conditions for in vitro primary intestinal epithelial cell and organ cultures. We will try to generate functional hedgehog and stromelysin-3 proteins and directly investigate the function of hedgehog and stromelysin-3 by adding over-produced proteins to organ or cell cultures. Alternatively, we can use antibodies to block the function of these proteins and examine the response of the cells or organs to TH. The analysis of the intestinal remodeling both in vivo and in vitro will include cell death, cell proliferation, and cell differentiation.

The complex nature of ECM and ECM remodeling will eventually demand the understanding of how different MMPs and ECM components participate in tissue remodeling. Thus, in addition to investigate the expression and biological function of MMPs during development and in organ cultures, biochemical studies will be carried out. Therefore, we will study the biochemical characteristic of ST3 in conjunction with our studies on Col4.

Finally, we have shown that primary epithelial cells cultured in vitro undergo apoptosis in response to TH. We will investigate the apoptotic mechanisms by studying marker gene expression during this process and the effects of various inhibitors that are known to block different steps of mammalian cell death. We also plan to investigate how various ECM affect epithelial cell behavior in vitro.

Significance to biomedical research and the program of the Institute

While metamorphosis is unique to lower organisms, the basic developmental process is similar to that in mammals. This is especially true for post-embryonic organ development. The regulation of the process by TH offers an unique opportunity to identify the genes which are important for organ development. The similarity in the function of organs and in their developmental processes between frogs and mammals suggest that the same genes which are involved in metamorphosis will likely be important for mammalian organogenesis. In addition, gene regulation by hormones is present in all vertebrate organisms. TH regulation of metamorphosis is an extreme example of hormonal regulation during development. Understanding the mechanism of how TH controls the transcriptional state of target genes, especially in a chromatin context, will be of general interest to researchers in the fields of hormones, transcriptional regulation and the effects of chormatin on gene expression.

Publications:

Ishizuya-Oka A, Ueda S, Shi Y-B. Transient expression of stromelysin-3 mRNA in the amphibian small intestine during metamorphosis. Cell Tissues Res 1996;283:325-329.

Kiang JG, Shi Y-B. Commentary: The third annual joint scientific symposium of NIH/FDA CAA and Washington DC Chapter of SCBA: Advances in Biological Sciences. J Biomedical Science 1996;3:133.

Puzianowska-Kuznicka M, Shi Y-B. Nuclear factor I as a potential regulator during post-embryonic organ development. J Biol Chem 1996;271:6273-6282.

Shi Y-B. Thyroid hormone-regulated early and late genes during amphibian metamorphosis. In Metamorphosis: Post-embryonic reprogramming of gene expression in amphibian and insect cells. (Eds. LI Gilbert, JR Tata and BG Atkinson) pp. 505-538. Academic Press, New York.

Shi Y-B, Ishizuya-Oka A. Biphasic intestinal development in amphibians: Embryogenesis and remodeling during metamorphosis. Current Topics in Developmental Biology 1996;32:205-235.

Shi Y-B, Stolow MA, Puzianowska-Kuznicka M, Wong J. Les genes de la metamorphose. La Recherche (French) 1996;286:58-64.

Shi Y-B, Wong J, Puzianowska-Kuznicka M, Stolow MA. Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: Roles of thyroid hormone and its receptors. BioEssays 1996;18:391-399.

Shi Y-B, Wong J, Puzianowska-Kuznicka M. Thyroid hormone receptors: Mechanisms of transcriptional regulation and roles during frog development. J Biomedical Science (in press).

Wong J, Shi Y-B, Wolffe AP. A role for nucleosome assembly in both silencing and activation of the Xenopus TRβA gene by the thyroid hormone receptor. Genes and Development 1995;9:2696-2711

Project Number: Z01 HD 01901-02 LME

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD01902-02 LME

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of the S phase checkpoint in higher eukaryotes.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Mary Dasso, Ph.D.	Unit Head	LME/NICHD
Others:	Hisato Saitoh, Ph.D.	Visiting Associate	LME/NICHD
	Kathy Steinmann, Ph.D.	Senior NRC Fellow	LME/NICHD
	Robert Pu, Ph.D.	IRTA Fellow	LME/NICHD
	Margaret Cavenagh.	Technician	LME/NICHD

COOPERATING UNITS *(if any)*

Dept. of Cell Biol., Johns Hopkins Med. Sch., Baltimore, MD (W. Earnshaw, Ph.D.); Dept. of Mol. Biol., Holland Laboratories of the American Red Cross, Rockville, MD (W. H. Burgess, Ph.D.); Dept. of Biochem., New York Univ. Med. Center, New York, NY (M. Rush, Ph.D.)

LAB/BRANCH

Laboratory of Molecular Embryology

SECTION

Unit on Cell Cycle Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-5430

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.6	4.0	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ran is a small, nuclear Ras-like GTPase that is required for nuclear transport, RNA transcription and processing, DNA replication, and cell cycle control. We have taken two approaches to the Ran GTPase pathway in *Xenopus* egg extracts: Purifying proteins that interact with Ran in a simple affinity assay, and cloning *Xenopus* homologues of known Ran-interacting proteins (Rips) in order to analyze their function *in vitro*. Using the first approach, we found that Ran associates specifically with at least ten extract proteins. We determined the identities of six Rips, and found that they include nuclear pore proteins and transport factors. We have examined the specificity of these interactions with respect to the guanine nucleotide bound to Ran and analyzed the interactions between endogenous Ran and Rips. We used the second approach to examine the role of RanBP1, a ubiquitous, highly conserved Ran binding protein, in nuclear assembly, transport and DNA replication. To do this, we removed RanBP1 from egg extracts by immunodepletion, and used the depleted extracts in nuclear assembly assays. We observed that RCC1, Ran's guanine nucleotide exchange factor (GEF), was unexpectedly co-depleted in these experiments. Our results suggest that neither RanBP1 nor RCC1 is essential for interphase nuclear functions in the absence of the other protein, but that the balance of RCC1 and RanBP1 is critical for proper nuclear assembly and function *in vitro*. Our work in *Xenopus* extracts is complemented by an analysis of S phase cell cycle controls in mammalian cells and by a search for the molecular target(s) of these controls. In particular, we have found mRNAs whose stability is controlled by the replication state of the nuclear DNA and we are in the process of characterizing these messages.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives: The objective of this unit is to discover the mechanism by which cells transmit and interpret signals that coordinate nuclear activities with each other and with the cell cycle. In particular, we have focussed on understanding the control of cell division with respect to the completion of DNA replication, since mitosis is inhibited by signals from the nucleus until DNA synthesis is complete. We are pursuing two molecular approaches to this problem: First, we are investigating the Ran GTPase pathway, which is required for nuclear transport and for coordination of nuclear activities to the cell cycle. Second, we are characterizing cellular mRNAs whose stability is regulated by the S phase checkpoint, and whose protein products may be required for the initiation of mitosis.

Methods Employed: *In vitro* biochemical studies of protein function and nucleic acid manipulation; functional investigations of Ran GTPase pathway components in *Xenopus laevis* egg extracts; Molecular biological analysis of mRNAs in mammalian tissue culture cell lines.

Major Findings:

1. Ran-associated protein in *Xenopus* extracts.

Table 1: Ran Interacting Proteins in *Xenopus laevis* egg extracts

Rip	molecular weight (kDa)	identity	binds GST-RCC1	co-ppt with Rip A
A	340	RanBP2	+*	
B	180	Nup153	-	-
C	120	?	-	-
D	92	Importin β	-	+†
E	88	RanGAP1-related	+/-	+**
F	72	hsc70	+*	-
G	45	RCC1		+††
H	30	RanBP1	+*	-
I	18	?	+/-	+**
J	11	?	-	-

*Associates with GST-RCC1 in the presence of EDTA. †Co-precipitates with RipA in the presence of GTPγS. **Co-precipitates with RipA in the presence or absence of guanine nucleotide analogs and EDTA. ††Co-precipitates with RipA only in the presence of EDTA.

We have been interested in understanding the nuclear roles of Ran. Mutants that interfere with Ran or RCC1, Ran's guanine nucleotide exchange factor, have a broad spectrum of defects *in vivo* that include abnormalities in nuclear transport, cell cycle progression, nuclear structure and RNA metabolism. It has been unambiguously demonstrated that Ran is required for nuclear protein import in a permeabilized cell assay, but it is currently unresolved whether the pleiotropic defects observed in these mutants exclusively reflect nuclear transport deficiencies or whether Ran may have multiple functions in the cell. In either case, an understanding of the Ran GTPase pathway will be essential for understanding the fundamental cell biology of the nucleus.

In order to gain a better understanding of the Ran GTPase pathway, we have used *Xenopus* egg extracts for the purification and characterization of pathway components. We have used a bacterially expressed glutathione S transferase-Ran fusion protein (GST-Ran) as a reagent in a rapid and simple affinity purification procedure, and found that GST-Ran associates specifically with at least ten extract proteins. We determined the identities of six Ran interacting proteins (Rips), and examined the specificity of their interactions with respect to Ran's nucleotide binding state. These Rips include: RanBP2/Nup358, Nup153, Importin β, hsc70, RCC1, and RanBP1. RanBP2 and Nup153 are nuclear pore proteins, and Importin β is required for targeting transport substrates to the nuclear pore. On the basis of peptide sequence, a seventh Rip (p88) appears to be similar but not identical to Fug1/RanGAP1, the GTPase activating protein (GAP) for Ran. The specificity of this method is supported by the fact that a number of the Rips have previously been found to interact with Ran in other assays (RanBP1, RCC1, Importin β, RanBP2), or to be constituents of the nuclear pore or to have roles in nuclear transport (hsc70, Nup153). To extend these observations, we examined the behavior of endogenous Ran and Rips in egg extracts, in order to learn how these proteins behave in the context of a complex mixture of proteins resembling that found in the intact embryo. Our data suggest that Ran associates tightly with Importin β in the presence of non-hydrolyzable GTP analogs, and that both proteins show increased association to RanBP2 under these conditions. RanBP2 is constitutively associated with p88, suggesting a possible model wherein nucleotide hydrolysis by Ran in the context of a Ran-Importin β-RanBP2-p88 complex may facilitate protein import.

2. RanBP1 and RCC1 act antagonistically in nuclear assembly and in nuclear transport.

Figure 1: RCC1 and RanBP1 act antagonistically in nuclear assembly and function. Equal volumes of Buffer (1), recombinant RCC1 (2), recombinant RanBP1 (3), or both RCC1 and RanBP1 (4) were added to co-depleted nuclear assembly reactions. A rhodamine-labelled import substrate was added at 60 minutes, and each sample was examined at 120 minutes. Phase contrast images of typical nuclei are shown (left), corresponding photographs of nuclear DNA (stained with Hoechst 33258) and protein import are shown in the middle and right columns, respectively.

RanBP1 was among the first proteins found to interact with Ran, through screening of a mouse cDNA expression library for proteins that bind to Ran-GTP. Like Ran, RanBP1 is ubiquitously expressed and highly conserved between species. RanBP1 is encoded by an essential gene in yeast, and it has been found to function as a guanine nucleotide dissociation inhibitor (GDI) for Ran-GTP *in vitro*. However, it remains mechanistically unclear how RanBP1 promotes nuclear transport because it is not required *in vitro* for protein import in permeabilized tissue culture cell assays. As an alternative approach to understanding RanBP1's biological role, we wished to use *Xenopus* egg extracts to develop an *in vitro* assay for RanBP1's function in transport and other interphase nuclear activities.

In order to examine RanBP1's role in nuclear assembly, transport and DNA replication, we cloned a *Xenopus* RanBP1 homologue and used it to generate recombinant RanBP1 protein and anti-RanBP1 antibodies. We removed RanBP1 from egg extracts by serial immunodepletions using affinity purified antibodies coupled to protein A Sepharose beads. Surprisingly, immunodepletion of RanBP1 from *Xenopus* egg extracts resulted in co-depletion of RCC1, suggesting that these proteins can form a stable complex in extracts. These extracts were examined for their ability to sustain nuclear assembly, DNA replication and nuclear transport. Extracts lacking both RanBP1 and RCC1 (co-depleted extracts) did not exhibit defects in any of these assays. Nuclei from co-depleted extracts also responded normally to the addition of recombinant cyclin B protein, undergoing nuclear envelope breakdown and chromatin condensation that was indistinguishable from that seen in control reactions. Addition of either recombinant RanBP1 or RCC1 to co-depleted interphase extracts caused abnormal nuclear assembly, inhibited nuclear transport and blocked DNA replication in a manner that could be rescued by further addition of RCC1 or RanBP1, respectively. These results suggest that neither RanBP1 nor RCC1 is essential for interphase nuclear functions in the absence of the other protein. The results further suggest that the balance of RCC1 and RanBP1 is critical for proper nuclear assembly and function *in vitro.*

3. Analysis of mutant Ran proteins in *Xenopus* extracts.

In order to examine the interactions between Ran and its effectors, a putative Ran effector mutant was generated in the laboratory of our collaborator, Dr. Mark Rush. This mutant was made by analogy to a Ras effector mutant at threonine residue 35 that acts abnormally with Ras effector proteins. The homologous residue in Ran is residue 42, which was mutated to an alanine. Ran(T42A) binds to GTP and responds normally to RCC1 as a nucleotide exchange factor in *in vitro* assays with purified proteins. However, it does not appear to be sensitive to GTPase stimulation by RanGAP1 in assays with purified proteins. Ran(T42A) had a reduced capacity to restore nuclear structure, nuclear protein import and DNA replication in RCC1-depleted extracts. We examined which of the Rips associated with Ran(T42A) in the context of *Xenopus* extracts. We found Ran(T42A)'s interactions with Importin β were substantially reduced, and we saw no interactions between Ran(T42A) and p120. RanBP1 also showed a lower affinity for Ran(T42A) than for wild-type Ran. Interactions with other Rips were at levels comparable to those observed with wild-type Ran. On the basis of these results, we believe that the interactions between Importin β, p120, RanBP1 and Ran are essential for accomplishing Ran's role in nuclear assembly, nuclear protein import and DNA replication.

4. The regulation of mRNA stability in the cell cycle.

Despite the importance of the DNA replication checkpoint for maintaining genetic stability in eukaryotic cells, many of the intermediary components in the checkpoint pathway coupling the onset of mitosis to the completion of DNA replication remain to be identified. One mechanism by which mitotic onset is controlled is through changes in mRNA stability. In BHK cells, at least some of the mRNAs required for mitosis are unstable as long as DNA replication is actively occurring. These mRNAs become stable if DNA replication is blocked by drugs that inhibit DNA replication (*e.g.*, hydroxyurea). Under these conditions, the cells are competent to enter mitosis in response to the addition of drugs such as caffeine. When the replication inhibitors are removed and DNA replication resumes, the mRNAs are rapidly destabilized and the cells lose their competence to enter mitosis in response to caffeine. These properties have allowed the identification of regulated mRNAs by the technique of differential display.

Figure 2: Destabilization of RNA G1-2 by ongoing DNA replication. The G1-2 band was excised from the S phase arrest lane of the display gel, re-amplified, random-primed and used to probe a Northern blot of the same RNA used for display. RNAs compared were from G1 phase arrested cells (G1); S phase arrested cells in hydroxyurea (S); cells released form S phase arrest for 15 minutes (R); G2 enriched cells (G2); and mitotic cells (M). Hybridization to a cDNA probe for the ribosomal protein S26 was used to normalize G1-2 expression levels for variation in the amount of total mRNA loaded per lane. Quantification was performed using a Molecular Dynamics PhosphoImager system.

To date, we have screened approximately half of the mRNAs present in interphase BHK cells by differential display for their patterns of stability. In this screen, we have found 23 mRNAs whose display patterns conform to that expected from messages whose stability is controlled by ongoing replication. Of these, six have been screened by Northern blot analysis. Those mRNAs whose abundance does not allow screening by Northern blot analysis will be screened using an RNase protection assay. Each of the mRNAs that give appropriate signals in the Northern blot analysis will be examined to determine their half-lives in the presence or absence of ongoing replication.

Proposed Course of Project:

1. Further characterization of Ran-interacting proteins (Rips) in *Xenopus*.

   Among the proteins that we found in association with GST-Ran, two have not been identified: p120 and p11. We believe that p120 and p11 are *Xenopus* homologues of proteins that have also been found in other organisms. In mammalian systems, other researchers have observed a 115/120 kDa protein in association with Importin β and GTP-Ran. On the basis of its similar behavior, it is likely that p120 is a *Xenopus* homologue of mammalian p115/120. It is likely that p11 is the *Xenopus* homologue of p10/NTF2, a 15 kDa protein that associates with GDP-Ran, based on their similar molecular weights and Ran-binding behaviors. During the coming year, we intend to confirm the identification of p120 and p11, and to investigate their interactions with Ran further using the *Xenopus* egg extract system. We have obtained peptide sequence information from p18, and we have cloned this protein by RACE-PCR using *Xenopus* oocyte cDNA as a template. We have raised antibodies against p18 and are pursuing studies related to its association with other Rips (see below).

On the basis of protein sequence analysis we have suggested that another of the Rips, p88, may be a GAP for Ran. However, we suspect that there may be multiple Ran GAPs in *Xenopus* eggs for two reasons: First, the four final amino acids corresponding to one of the p88-derived peptides that were sequenced are absolutely conserved in Fug1/RanGAP1 sequences from humans to yeast (VINLDNDNTF), so their lack of conservation in p88 (VINLDNDLAE) may indicate that it is divergent from Fug1/RanGAP1 proteins. Second, we have cloned a partial cDNA for a *Xenopus* Fug1/RanGAP1 homologue that encodes a protein with approximately 80% conservation to human RanGAP1 through the first 361 amino acids of the human sequence. The corresponding peptide sequence in this cDNA is the same as those in the mammalian Fug1/RanGAP1 cDNAs. This cDNA also does not encode four additional peptide sequence that we obtained from p88.

We are taking two approaches to examine whether there are multiple Ran GAPs in *Xenopus*: First, we are examining p88's enzymatic activity as a GAP for Ran, and second, we are performing cloning experiments to obtain complete sequence information and determine whether multiple GAPs for Ran exist in *Xenopus* eggs. Preliminary analysis indicates that p88 does indeed act as a GAP for Ran, and we are currently investigating how the activity of p88 is regulated (see below). We have recently obtained a full-length cDNA for *Xenopus* RanGAP1. We are now in the process of determining whether p88 and RanGAP1 are distinct proteins, or whether p88 is produced by alternative splicing of RanGAP1 RNA or post-translational modification of RanGAP1 protein. In the latter case, we will pursue the nature of the modifications on p88 and how they may change the protein's localization or activity.

2. Use of *Xenopus in vitro* systems for analysis of Ran GTPase pathway components.

The analysis of Ran pathway components in *in vitro* systems will focus on two aspects of Ran function: First, the establishment of assays for pathway components through their immunodepletion. The establishment of such assays will allow the investigation of functional domains of the proteins through mutagenic studies. Second, we will investigate the interactions between pathway components in egg extracts, concentrating on the p340-p88-p18 complex.

We have been able to determine conditions under which nuclear assembly and protein import are dependent on the addition of exogenous RanBP1, allowing *in vitro* analysis of RanBP1's role in these processes. We have also generated mutants in RanBP1 that will be expressed in bacteria and purified. By determining which mutants are capable of restoring nuclear assembly and protein import to depleted extracts, we will define the critical domains of RanBP1 required for its activity. These mutants will then be used as affinity reagents in experiments similar to those already discussed for Ran. The combination of these two approaches may simultaneously provide evidence of which parts of RanBP1 are required for its function and of what other extract proteins interact with these domains. We have generated antibodies against *Xenopus* RanGAP1, and are currently attempting to establish a similar *in vitro* approach using extracts lacking RanGAP1.

We have generated antibodies that recognize p340, p88 and p18. Using these reagents, we have been able to demonstrate several important properties of these proteins: First, we have found that a complex containing each of these proteins can be immunoprecipitated with antibodies recognizing any one of them. Second, we have found that immunoprecipitates of this complex have GAP activity for Ran. We have already established that p340 is localized to the cytosolic face of the nuclear pores. Given this localization and data from other laboratories suggesting that GTP hydrolysis by Ran occurs on the cytosolic face of the nuclear pore, it will be important to determine whether the formation of this complex is critical for Ran's role in nuclear import. In the immediate future, we will use the antibodies that we have generated to determine whether p340, p88 and p18 all co-localize in vitro after the assembly of nuclear envelopes and in vivo. We will also examine whether this complex is stable in all phases of the cell cycle and whether any of the components is subject to post-translational modification. Eventually, we would like to examine whether the formation of this complex has any regulatory role in controlling nuclear transport ~~of~~ or other aspects of Ran function. In order to do this, we will attempt to biochemically separate p340, p88 and p18 from each other and to reconstitute Ran GAP activity and in vitro nuclear assembly in the absence of each of the proteins in order to establish assays for their function.

3. Systems regulating mRNA stability in the cell cycle.

We are continuing to screen candidate mRNAs for the regulation of their stability in response to ongoing DNA replication. At the same time, we will isolate full-length cDNAs for the messages whose stability we have demonstrated to be regulated. We then plan to find regions of the mRNAs that confer instability. To do this, the cDNAs corresponding to these mRNAs will be expressed from expression vectors in BHK cells. We will confirm that the mRNAs expressed from these vectors are regulated in a manner similar to the endogenous mRNA, then we will map the instability region by deletion analysis. We will also examine the behavior of stability-regulated mRNAs under other conditions where the S phase checkpoint fails. Namely, we will examine how these mRNAs are regulated in tsBN2 cells, a temperature-sensitive BHK cell line with a mutant allele of RCC1. S phase blocked tsBN2 cells advance precociously into mitosis at the restrictive temperature, regardless of the replication state of their DNA. It will be of interest to make a comparison at the molecular level between caffeine-induced premature mitosis and premature mitosis in tsBN2 cells.

After the sequences responsible for mRNA stability are determined, we will pursue two avenues of experimental investigation: First, we will seek to demonstrate what role the proteins encoded by these mRNAs play in the regulation of mitosis. A direct role in caffeine-induced premature mitosis could be demonstrated if premature mitosis does not occur when the expression of a particular protein is blocked through antisense RNA strategies or if premature mitosis is potentiated when we express a message lacking the mRNA destabilizing element. Proteins that override the DNA replication checkpoint control when inappropriately expressed would be expected to interact with or be key components of the checkpoint pathway. Second, we would seek to identify the mechanisms through which the destabilizing sequences confer regulation on these mRNAs. For instance, mRNA stability could be controlled by the regulated binding of a protein to the stability sequence. We would test whether this is also the case for checkpoint regulation of mRNA stability, and if so, we would seek to discover the identity of the binding protein and how it is regulated by the checkpoint pathway.

Significance to biomedical research and the program of the Institute:

Orderly progression through the cell cycle is required for the fidelity of transmission of genetic material. Cell cycle controls insure that initiation of later cell cycle events depends on the completion of earlier events. For reasons that are discussed above, we have chosen two points at which to investigate the S phase checkpoint: The Ran GTPase pathway and the regulation of mRNA stability. Investigations of the Ran GTPase pathway will contribute to our knowledge of the fundamental cell biology of the nucleus and nuclear transport. Eventually, we anticipate that we will find how these essential nuclear activities are related to the signals that are communicated between the nucleus and the cytoplasm in order to control the cell cycle. Increasing our knowledge of how this checkpoint operates will ultimately help determine whether DNA replication checkpoint failure contributes to disease states such as cancer. If so, this understanding of the cell cycle may contribute not only toward basic biological knowledge but also toward the development of diagnostic tests and treatments for cancer.

Publications:

Dasso, M. (1995). The role of the Ran GTPase Pathway in Cell Cycle Control and Interphase Nuclear Functions. *Prog. Cell Cycle Res.*, **1**, 163-172.

Drivas, G., Villamarin, A., Murphy, G., Moore, M.S., Dasso, M., D'Eustachio, P. and Rush, M.G. (1996). A T42A Ran Mutation: Differential Effects on Nuclear Protein Import, Nuclear Structure, and DNA Synthesis. (submitted.)

Pu, R. T. and Dasso, M. (1996). RanBP1 and RCC1 act antagonistically in nuclear assembly and nuclear transport: Studies in *Xenopus laevis* egg extracts. (submitted.)

Saitoh, H., Cooke, C. A., Burgess, W. H., Earnshaw,W. C. and Dasso, M. (1996). Direct and Indirect association of the small GTPase Ran with nuclear pore proteins and soluble transport factors: Studies in *Xenopus laevis* egg extracts. *Mol. Biol. Cell*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD01903-01 LME

PERIOD COVERED

July 8, 1996 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of SMC-proteins and other chromosomal proteins in mitotic chromosome segregation.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and Institute affiliation)*

PI:	Alexander V. Strunnikov	Unit head	LME:NICHD
Others:			

COOPERATING UNITS *(if any)*

N/A

LAB/BRANCH

Laboratory of Molecular Embryology

SECTION

Unit of Chromosome Structure and Function

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
.25	0.25	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The proper chromosome transmission in mitosis is facilitated by numerous cellular factors. Particularly, chromosome proteins are actively engaged in mitotic segregation of chromosomal material. The newly discovered family of chromosomal proteins, the SMC family, represents an important subset of these polypeptides. The SMC-proteins are present in most of the cellular organisms. Their importance is especially profound in the process of chromosome condensation during the mitotic prophase. They also are indispensable for the disjoining of sister chromatids during the anaphase. Thus this family is one of the central players in the mitotic chromosome segregation. The goal of the ongoing studies is to elucidate the molecular mechanics behind the activity of these proteins in mitosis. This work follows four major directions. First, structure-function analysis of the SMC molecules, beginning with assigning a biochemical activity or a cellular function to the putative domains of the given SMC-protein. These studies include mutational analysis of four SMC genes in bakers yeast (SMC1, SMC2, SMC3, SMC4) and development of the system for allele replacement of mouse smc genes in cultured cells. Second, cell cycle specific distribution and regulation of the SMC-proteins is being assessed through the studies of their subcellular localization. For that purpose the polyclonal antibodies are being generated against all yeast SMC proteins, and the monoclonal antibodies against human SmcA and SmcB are at the stage of development. Third, the in-vitro assay for chromosome condensation employing a controlled number of purified components is being developed. This in-vitro system is necessary to assess the actual molecular function of the SMC-proteins. Fourth, we conduct a biochemical and genetic search for the proteins interacting with the SMC proteins, both in yeast and mammalian cells. Three proteins interacting with the SMC function in yeast have already been identified.

Project Description:

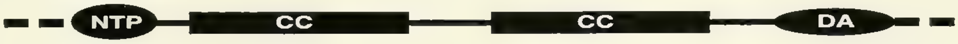
Objectives: To understand a role of SMC proteins and other chromosomal proteins in mitotic chromosome segregation. The discovery of the SMC proteins is an important advance in the understanding of chromosome structure. This is a new group of chromosomal proteins that are indispensable for chromosome segregation. In contrast to histones, HMG proteins and topoisomerase II, the SMC proteins appear to be mainly involved in mitotic chromosome condensation. The importance of this group became evident just recently, after a number of corresponding genes have been cloned and their products characterized from a variety of organisms, including bacteria, fungi , invertebrate and vertebrate animals.

Members of the SMC family range from 115 KD to 160 KD in their molecular mass, and can be subdivided into at least 4 structurally distinct subgroups. The amino acid sequence of a typical SMC protein predicts five major regions: an NTP-binding region, the first stretch of the coiled-coil alpha helix, a "hinge", the second coiled-coil region, and a COOH-terminal region harboring the DA-box, a signature motif for the SMC family (Figure 1).

Most of the in-vivo data regarding the function of SMC proteins in mitosis came from the studies of fungi. In *S. cerevisiae* four structurally distinct SMC proteins are known : Smc1, Smc2, Smc3 and Smc4. Remarkably, in this organizm three SMC proteins are indispensable for cell viability. Thus, it appears that multiple types of the SMC proteins in the same cell have at least some essential nonoverlapping functions.

To elucidate the functions of SMC proteins that are important for chromosome segregation we plan our work in four major directions. First: structure-function analysis of the SMC molecules. Second: cell-cycle-specific distribution and regulation of the SMC proteins. Third: development of the in-vitro assay for chromosome condensation employing only purified components. Fourth: biochemical and genetic search for the factors interacting with the SMC proteins

A.



B.

```
Smc1   GRLVGLELSN FKSYRGVTKV GFGE*NFTSI IGPNGSGKSN MMDAISFVLG VRS*KLVELM
Smc2   MKVEELIIDG FKSYATRTVI TDWD*QFNAI TGLNGSGKSN ILDAICFVLG IAS*PQISVT
Smc3   MYIKRVIIKG FKTYRNETII DNFS*HQNVI IGSNGSGKSN FFAAIRFVLS DDY*DDEVTI

Smc1   RIISRNGDTS YKIDGKTVSY KDYSIFLENE NILIKAKNFL VFQGDVEQIA ..........
Smc2   RQVVLGGTSK YLINGHRAPQ QSVLQLFQSV QLNINNPNFL IMQGKITKVL ..........
Smc3   RRTVGLKKDD YQLNDRNVTK GDIVRMLETA GFSMNNPYNI VPQGKIVALT ..........

Smc1   *ELAGGNASL TIEDEDEPFN AGIKYHATPP LKRFKDMEYL SGGEKTVAAL ALLFAINSYQ
Smc2   *DLLPNSFAK LVPCEGKDVT QGLEVKVKLG NIWKESLIEL SGGQRSLIAL SLIMALLQFR
Smc3   *DAESNESQN GKDSEIMYTG VSISVSFNSK QNEQLHVEQL SGGQKTVCAI ALILAIQMVD

Smc1   PSPFFVLDEV DAALDITNVQ RIAAYIRRHR
Smc2   PAPMYILDEV DAALDLSHTQ NIGHLIKTRF
Smc3   PASFYLFDEI DAALDKQYRT AVATLLKELS
```

Figure 1. **A.** Scheme of the putative structure of an SMC protein. NTP - putative NTP-binding region; CC - coiled coil regions; DA - DA-box region. **B.** Amino acid sequence alignment of the conserved regions of three yeast SMC proteins (Smc4 is not shown).

Major Findings: N/A

Proposed Course of the Project:

1. Structure-function analysis of the SMC molecules. This study will allow to assign a biochemical activity or a cellular function to the putative domains of a given SMC protein. These experiments include mutational analysis of four SMC genes in bakers yeast (SMC1, SMC2, SMC3, SMC4). The DA-box and the NTP-binding region of the SMC proteins are thought to be involved in the ATP hydrolysis. Deletions of these motifs from the Smc1 protein have already demonstrated that they are essential for the function of Smc1. This finding is to be strengthened by the introduction of point mutations into these regions. Several conditional lethal mutations have been mapped to the coiled-coil regions, thus showing the importance of these potential protein-protein interaction domains for the SMC proteins. We are going to shuffle the coiled coil domains between four yeast SMC proteins in order to determine their degree of specificity. Employing the bakers yeast as a model system for these studies has an obvious advantage of combining genetic, molecular and cytological techniques in the same organism. All four yeast SMC genes are already cloned and sequenced. Yeast chromosomes, however do not represent a typical eukaryotic chromosome, mainly due to their short length and the low degree of condensation in mitosis. In that regard chromosomes of mammalian cells are much more attractive for the detailed investigation of the SMC proteins role in mitosis. We plan to apply methodology of the structure-function analysis developed with yeast SMC proteins to the mammalian cells. Particularly, we plan to create an embryonic stem cell line with a given SMC gene deleted from its proper chromosomal location, thus allowing studies of the mutant forms of mouse or human SMC cDNAs, expressed ectopically. This project requires cloning of the full length cDNAs of smcA, smcB and smcD genes, using fragments of their cDNAs as molecular probes.

2. Cell cycle specific distribution and regulation of the SMC-proteins. Taking into account that eukaryotic cells contain more than one type (usually four) of the SMC proteins it is important to learn more about specificity of a given SMC protein. Particularly it is of great importance to understand differences in cell distribution of SMC-proteins and their possible specialization. For that purpose the polyclonal antibodies need to be generated against all four yeast SMC proteins, and the monoclonal antibodies against human SmcA and SmcB. Corresponding antigens are to be purified from E. coli cells overexpressing the fragments of SMC proteins. Then the antibodies will be used for microscopic studies of the mammalian and yeast cells using immunocytochemistry.

3. Development of the in-vitro assay for chromosome condensation. Despite the fact that an in vitro system for condensation of sperm chromatin in the crude amphibian egg extracts has been reported, the in-vitro assay employing a controlled number of purified protein factors has many attractive features. Particularly, if such system can be developed, it can help in elucidating the fine molecular mechanisms underlying the activity of SMC proteins in chromosome condensation. In order to develop such a system, the adequate substrate for chromosome condensation (interphase chromatin) need to be combined with the appropriate

protein factors, possessing a chromosome condensing activity. SMC proteins are good candidates to bear that activity. Thus, development of a protocol for purification of the SMC proteins in their active form is considered a high priority.

4. Biochemical and genetic search for the factors interacting with the SMC proteins. It is highly unlikely that SMC proteins perform their chromosomal function on their own, without a significant interaction with other chromatin components. Identification of the SMC partners could serve two major goals: to establish a link to the well-known proteins, like histones or topoisomerases, and to reveal the presence of some new, previously unknown components. This work is to proceed in two directions. First, interacting proteins can be identified biochemically through micropeptide sequencing of the proteins, copurified with the SMC fraction; second, a number of genetics screens can be applied to find the SMC-interacting molecules (suppressor and synthetic phenotype analysis in yeast, two-hybrid screening against a human cDNA library). Three proteins potentially interacting with the Smc1 and Smc2 proteins in yeast have already been identified

Methods Employed:

Genetics: transformation of yeast cell, mutagenesis, two-hybrid screening, transfection of mammalian cells. Biochemistry: extractions, column chromatography, FPLC, ATP-ase assay, immunoprecipitation, chromatin assembly, gel-shift protein-DNA binding assay, in vivo footprint analysis. Cytology: fluorescent and DIC microscopy, digital image capture with cooled CCD camera, fluorescent in situ hybridization, immunocytochemistry. Molecular biology: DNA sequencing, PCR, Southern hybridization, gene cloning, site-directed mutagenesis.

Publications:

D. Koshland and A. Strunnikov. Mitotic chromosome condensation. Annual Reviews of Cell and Developmental Biology 1996;12:305-333.

LMG

LABORATORY OF MOLECULAR GENETICS
Igor B. Dawid, Ph. D., Chief

In the past year the members of the Laboratory have extended their studies on mechanisms of gene regulation in different systems and in different developmental and physiological conditions. These problems were explored for bacterial and animal viruses, in the responses of bacterial cells to environmental conditions, and during development of multicellular organisms. A particular focus of the research in the Laboratory is the question of pattern formation and its genetic control in different animals. In the recent past very substantial progress has been made in this area of research, allowing the study of classical question of embryology at the cellular and molecular level. Work in this Laboratory contributes to this progress in the areas of axis establishment, cell-cell signalling in mesoderm and neural induction, and the elaboration of regulatory circuits during embryogenesis.

AXIS ESTABLISHMENT IN THE VERTEBRATE EMBRYO

The molecular basis of early embryogenesis has been the focus of study in the group headed by *Igor Dawid*. The experiments used *Xenopus laevis* and the zebrafish as suitable experimental materials. In the past year work focused on two main topics, certain aspects of the role of Wnt factors in early development and the function of the LIM homeodomain protein Xlim-1 during gastrulation.

Use of the *Xenopus* embryo as an assay system for Wnt ligand-receptor interactions. The Wnt family of signaling molecules is involved in early pattern formation in several ways. This laboratory and others have shown previously that the Wnt signaling cascade regulates the initial dorsoventral polarization in the embryo. During the past year relationships between different Wnt factors and certain Frizzled proteins, recently implicated as Wnt receptors, were studied in collaboration with Xi He and Harold Varmus. Since some Wnt molecules can induce a dorsal axis but others cannot, the possibility that the receptors for the latter is missing was explored. The results showed that this is the case. While Wnt5A does not induce a dorsal axis, as previously reported by Moon and colleagues, the combined expression of Wnt5A and Frizzled 5 does. This result provides evidence that Frizzled 5 can act as a Wnt5A receptor, and further suggests that the difference in activity in the early embryo between Wnt5A and certain other Wnt factors (e.g., Xwnt8) can be explained by differential expression of their cognate receptors.

The role of Wnt factors in neural patterning. Various studies have shown that the initial neuralizing signal leads to the formation of anterior structures, some of which are later converted to more posterior ones. The mechanism of this conversion is thus an important question, and Wnt factors have been implicated in this mechanism by the group of R. Moon. Extending these observations, recent work in the Laboratory has shown that certain Wnt factors, e.g., Wnt3A and Wnt1, but not Xwnt8 or Wnt5A, strongly affect neural patterning. However, the results obtained further indicate that Wnt1 and Wnt3A do not so much posteriorize the neural plate as induce neural crest. The formation of the neural crest, which ultimatley gives rise to a vast array of cell types and tissues, is a problem of fundamental importance in vertebrate development. Finding a putative role for Wnt factors in this process promises substantial progress.

The LIM class homeodomain protein Xlim-1 collaborates with the Otx2 protein in the activation of the *goosecoid* gene. In earlier work, the *Xlim-1* gene has been implicated in neural and muscle induction, and it has shown that an activated form of Xlim-1 can stimulate the transcription of the homeobox gene *goosecoid* (*gsc*). More recently the mechanism of regulation of *gsc* by Xlim-1 has been studied in collaboration with K. Cho and colleagues, leading to the suggestion that the *gsc* gene is maintained in an inactive state by a repressor. While mutated Xlim-1 protein is an activator of *gsc*, the wild type protein cannot act by itself but is effective in combination with Otx2. The homeodomain protein Otx2 appears capable of displacing the hypothetical repressor, allowing the wild type Xlim-1 protein to activate the *gsc* gene. These studies begin to illuminate the regulatory interactions between transcription factor-encoding genes that lead to the patterning of the gastrula embryo.

Ldb-1, a LIM domain binding protein, functionally interacts with Xlim-1 in vivo. In collaboration with A. Agulnik and H. Westphal, a gene encoding a novel LIM domain-binding protein was isolated; it was named Ldb-1. Ldb-1 is highly conserved from mouse to *Xenopus* and zebrafish, with 95-97% identity over the entire length of the protein. Ldb-1 binds LIM domains from LIM-homeodomain proteins, but not from certain other, more distantly related LIM domain proteins. When coexpressed in the frog embryo, Ldb-1 acts synergistically with Xlim-1 in the induction of partial secondary axes and in the activation of target genes. Ldb proteins may be important regulators of the function of LIM class homeodomain proteins.

GENE REGULATION IN VERTEBRATE EMBRYOGENESIS

The general aim of the project conducted by *Tom Sargent* and his colleagues is to identify and study the function of factors that regulate vertebrate embryonic development. This is being approached in projects that deal with a study of cell-cell signalling and with an analysis of the function of a transcription factor of the homeodomain class in ectodermal specification.

The role of the Dlx3 protein in the formation of epidermis and other ectodermal derivatives. This approach consists of the analysis of the regulation and function of Dlx3, one of the vertebrate homologs of the *Drosophila* gene *Distal-less*. In flies this gene regulates limb and sensory appendage formation. The frog and mouse homologs are both expressed in epidermis, branchial arches, and in gradients in the limbs. The mouse gene is also active in hair follicles, tooth primordia and other locations. DNA elements and protein-DNA interactions responsible for this expression pattern are being mapped. In addition, targeted inactivation and mis-expression studies are being used to test for the function of *Dlx3* gene expression in the epidermis of transgenic mouse embryos. This laboratory discovered that ectopic *Dlx3* expression in basal keratinocytes alters the identity of these cells, partially transforming them into more differentiated keratinocytes. Phenotypic changes following Dlx3 misexpression include activation of the profilaggrin and loricrin genes, termination of cell division, reduction in hair follicle density and disruption of the epidermal barrier to water loss. These results suggest that *Dlx3* is critically involved in regulating the orderly course of epidermal differentiation.

The role of the receptor tyrosine kinase Pag in cell adhesion. A second project of Sargent's group has been to identify genes encoding Eph-related receptor tyrosine kinases that are expressed in specific tissues of early frog embryos. One such kinase, called Pagliaccio (Pag), has been shown to affect cell-cell adhesion when constitutively activated in *Xenopus* embryonic ectoderm. Activation of Pag leads to dissociation of blastula cells and interferes with gastrulation movements in ectodermal explants induced with activin. These effects are probably caused by modulating cadherin function. The basis of this suggestion is the fact that the embryonic dissociation elicited by activated Pag can be rescued by administration of additional cadherin to the cells by RNA microinjection.

REGULATION OF HOMEOTIC GENE EXPRESSIOIN IN *DROSOPHILA*

The study of *Drosophila* as a model system has been particularly effective in elucidating the genetic basis of pattern formation. The discovery of homeotic genes, which control pattern, and their detailed study has revolutionized biology in the recent past. Homeotic genes control pattern formation through their spatially and temporally regulated expression during development which in turn leads to regulated expression of multiple target genes. Thus the issue how the expression of homeotic genes is regulated is a major question in developmental genetics. ***Jim Kennison*** has studied this question for some time, by isolating genes that regulate the expression of homeotic genes through the use of a sophisticated genetic approach. In this approach a strain carrying a partially effective ("leaky") mutation in one homeotic gene was screened for independent mutations that enhance or repress the original mutant. In this sensitized system, genes that subtly affect the expression or function of the original gene could be discovered, leading to the identification of about a dozen novel genes that interact with certain homeotic and segmentation genes in *Drosophila.*

Regulatory hierarchies of homeotic genes. As part of efforts to characterize the function of regulatory genes previously identified as important in regulating expression of homeotic genes, the characterization of mutations interacting with *brahma* was carried out. The *brahma* gene encodes a nuclear protein that is conserved from yeast to man and is found in large complexes that include at least nine other polypeptides. The yeast homologue, SNF2/SWI2, is a DNA-dependent ATPase that is required for transcriptional activation of multiple target genes. Mutations were isolated in six other genes that interact with *brahma*, including the *osa* gene that Kennison had already shown was required for homeotic gene transcription. The *osa* gene has been isolated and is being characterized. Another of the interacting mutations, *E(brm)3*, was mapped by transposon tagging, allowing molecular characterization to proceed.

Studies on gene silencing. A group of *trans*-acting factors, the Polycomb group, are know to be required for maintenance of homeotic gene silencing. The initiation of silencing requires, among others, the function of the *hunchback* segmentation gene. In a collaboration with J. Mueller and M. Bienz, Kennison is studying the gentics of a factor that interacts with Hunchback in silencing. A candidate mutation that was isolated, *l(3)76BDo*, is a new member of the Polycomb group. This mutation and addition alleles will be important in a genetic dissection of silencing.

In a second approach to silencing, the role of repetitive sequences in silencing of genes in the Antennapedia complex was studied. It appears that specific sequence elements embedded in the repetitive DNA are responsible for silencing in this gene complex.

MOLECULAR GENETICS OF PROTEIN-NUCLEIC ACID INTERACTIONS IN DROSOPHILIA

RNA binding proteins have been identified and studied in many organisms. Their study in *Drosophila* has allowed the application of both biochemical and genetic tools, greatly aiding elucidation of their biological functions. The project directed by ***Susan Haynes*** aims to understand the roles of RNA binding proteins in the post-transcriptional regulation of gene expression in *Drosophila*, in particular to direct attention to functions in development and in cell type specific differentiation. Haynes and colleagues are approaching this issue through an analysis of the in vivo functions of proteins that contain so-called RNA recognition motifs (RRMs). Such domains occur in many proteins involved in post-transcriptional regulation, and can mediate sequence-specific binding to RNA targets.

Functions of hnRNP proteins in *Drosophila*. Three of the proteins studied by Haynes and colleagues, HRB87F, HRB98D and CAZ, are homologs of vertebrate proteins that bind nuclear pre-mRNA (hnRNP proteins). Mutations in both *Hrb* genes have been isolated and studied. The mutation in the *Hrb87F* gene completely deletes the coding region. Since homozygous mutant flies are viable and fertile, these observations demonstrate that the HRB87F protein is not essential. However, in the appropriate genetic background, these flies are sensitive to the dosage of HRB98DE. Using this observation, Haynes and colleagues performed a genetic screen and isolated a mutation in *Hrb98DE.* This mutation abolishes the expression of the protein, as shown by Western blots of protein extracts from adults. Homozygous or hemizygous individuals show somewhat reduced viability but, like HRB87F, HRB98DE is not essential. However, homozygosity for both mutations is lethal, demonstrating that flies require a minimal level of HRB proteins for survival and suggesting that the two HRB proteins are functionally redundant. In vitro studies have suggested that hnRNP A1, a vertebrate homolog of the HRBs, may be a general splicing regulator. Data from overexpression and reduced expression fails to support such a role for the HRB proteins. However, preliminary data suggest that in the appropriate sensitized genetic backgrounds, reduced *Hrb* gene dosage can modulate the expression of the *Ultrabithorax* gene, which regulates segment identity. The CAZ protein, the *Drosophila* homolog of hnRNP P2, shows widespread but not ubiquitous expression during development. Haynes and colleagues identified a novel RRM-containing protein that interacts with the CAZ protein in a yeast two-hybrid selection screen, and that may be a specific partner in ribonucleoprotein complexes.

The function of the *Tsr* and *Rb97* genes in spermatogenesis. The *Tsr* gene encodes a cytoplasmic RRM protein that is expressed exclusively in the testis. In the absence of this protein, most sperm fail to complete development, and instead degenerate in the testis. Post-transcriptional regulation is very important during spermatogenesis, since most transcripts required after meiosis are synthesized premeiotically and stored as RNP complexes; thus a role for an RRM protein in spermatogenesis is biologically plausible. Experiments analyzing the time when TSR function is required suggest that TSR has a pre-meiotic function, and that TSR acts to repress the expression of one or a few proteins before meiosis. Like *Tsr, Rb97* gene function is required for spermatogenesis in *Drosophila*, and RB97 protein has been shown to bind to a region of the Y chromosome. The role of RB97 in affecting putative functions of this region of the chromosome are being studied.

MOLECULAR GENETICS OF MAMMALIAN RETROVIRUS REPLICATION

The goal of the project conducted by ***Judith Levin*** is to define the molecular mechanisms involved in the replication of mammalian retroviruses and in particular, to understand the factors which influence the regulated expression of viral genetic information. This question is clearly of great interest since the efficiency of viral DNA synthesis will have a direct effect on the efficiency of viral replication.

The role of nucleocapsid protein in viral replication. Levin and colleagues have used an in vitro approach to study events in reverse transcription and the role of the viral nucleocapsid protein (NC) in increasing the efficiency of viral DNA synthesis. They have shown that HIV-1

NC reduces reverse transcriptase (RT) pausing near the murine leukemia virus polypurine tract (PPT) during elongation of (-) strand DNA. Mutational analysis indicates that the features of HIV-1 NC which contribute to NC activity include: the basic residues flanking the N-terminal zinc finger, the zinc finger structures, and the cysteine and aromatic amino acids within both fingers.

Studies on first strand-transfer during HIV replication. Levin's group also developed an in vitro HIV-1 system to study the first strand-transfer, which occurs after synthesis of (-) strong stop (SS)DNA. Using donor and acceptor RNAs containing TAR (a large stem-loop in the terminal repeat region which could interfere with annealing of (-) SSDNA and acceptor), they observed that the products are largely heterogeneous (+) strand DNAs formed by a self-priming mechanism from (-) SSDNA; in this case, transfer efficiency is only 2%. Deletion of TAR or addition of NC greatly stimulated strand-transfer and drastically reduced self-priming. Thus, NC overcomes the potential inhibitory effect of TAR, while allowing the virus to maintain TAR for subsequent transactivating activity. Experiments to determine whether self-priming can be detected in endogenous reactions with HIV-1 virions are in progress.

Mutational analysis of the HIV "primer grip" region. Levin and colleagues have analyzed HIV-1 RT mutants having alanine substitutions in the "primer grip" region. These mutants can extend DNA primers, but most of the mutants cannot utilize RNA primers for initiation of (+) and (-) strand DNA synthesis; several mutants are also deficient in specific RNase H cleavage at the 3' terminus of the PPT. These studies led to the conclusion that the primer grip region plays a major role in recognition of primer-template structures containing the PPT or $tRNA^{Lys3}$ RNA primers. The results of these experiments contribute to the general understanding of the molecular control that are in play during retroviral, specifically HIV, replication.

COORDINATED GENE REGULATION IN E. COLI

Michael Cashel and his colleagues have continued their investigations of the mechanisms that control changes in *E. coli* gene expression during adaptation to the stress of transitions between nutritional impoverishment and abundance. The expression of the genomic repertoire of *E. coli* is coordinately regulated in response to nutrient availability. This determines growth as well as the ability to survive prolonged periods of starvation. The project pursued by Cashel and colleagues focuses on regulation of these processes in bacteria by analogs of the common nucleotides GDP and GTP that possess ribosyl 3' pyrophosphate esters, collectively known as (p)ppGpp. Intracellular levels of (p)ppGpp are altered in response to availability of amino acids, phosphate, nitrogen or energy; the (p)ppGpp signal, in turn, triggers globally altered patterns of gene expression. In addition, (p)ppGpp recruits global domains of gene expression associated with stationary phase. Thus, a broad repertoire of physiological responses is being investigated through his approach.

Mutational analysis of (p)ppGpp regulation. This year Cashel and colleagues have found evidence of recruitment of cell cycle regulation domains and gene domains responding to the leucine regulatory protein (lrp) gene. Current evidence arising from mutant analyses is beginning to define the extent of global regulatory domains directly or indirectly regulated by (p)ppGpp. So far, mutants that phenotypically mimic the presence of (p)ppGpp, when this nucleotide is actually absent, are found to occur only in three RNA polymerase subunit genes. Previously two "housekeeping" sigma mutants have been decribed and their effects on premature RNA chain release during transcription initiation on model promoters has been studied. This year these effects are extended to suggest that physiologically relevant promoters responsible for synthesis of galactose or histidine are stimulated by the mutants by increasing promoter clearance efficiency. Attention is now given to other RNA polymerase mutants. Cashel and colleagues have mapped and identified the sequence changes for 59 different alleles of genes encoding the two large "core" RNA polymerase subunits (beta and beta'). In all, 52 new alleles have been documented. There is early evidence that these mutants do not regulate galactose promoters in the same way as previous sigma mutants. They are therefore of great interest.

RECOMBINATION AND TRANSCRIPTIONAL CONTROL IN LAMBDOID PHAGES

Bacteriophage λ and its relatives have long been prime models for the study of basic molecular mechanisms. ***Robert Weisberg*** and his colleagues have used this system to great advantage in their continuing studies on the mechanisms of recombination and transcription. This model continues to allow the most fundamental and penetrating studies on molecular mechanisms common to a variety of other systems.

Regulation of transcription by antitermination. Antitermination increases the transcription of genes that are located downstream of terminators. While best studied in bacteriohages, this mechanisms also occurs in other viruses and may be of more general importance. Transcripts encoded by the cis-acting antitermination sites (*put* sites) of lambdoid phage HK022 promote readthrough of downstream transcription terminators. Proper conformation of the transcripts is essential for activity, since *put* mutations that prevent the formation of predicted RNA stems prevented antitermination, and suppressor mutations that restore the stems restored antitermination. Antitermination does not appear to require proteins other than RNA polymerase, since *put*-dependent readthrough of multiple sequential terminators was observed in a purified transcription system containing only template, polymerase, substrates, buffer, and product. Transcription of *put* also increased the elongation rate of polymerase, very likely by suppressing pausing. A mutation that alters the zinc-finger region of the β′ subunit of polymerase specifically prevented the *put*-dependent increases in terminator readthrough and elongation rate. The simplicity of HK022 antitermination contrasts with that of other known antitermination pathways. Weisberg and colleagues propose that the central effector is a transcript that directly alters the elongation properties of RNA polymerase.

Specificity of interaction between integrase and its cognate sequences. The integrase proteins of phages λ and HK022 are closely related site-specific recombinases that recognize different nucleotide sequences in the core regions of their substrates, the attachment sites of the two phages. Several attachment site nucleotides that inhibit recombination by the non-cognate integrase have now identified. These include G4 in the λ B' core site and A1 and C3 in the HK022 B' and C core sites. Interestingly, nucleotides flanking the canonical core sites also appear to be recognized by integrase. These studies illuminate molecular mechanisms of a specific aspect of genetic recombination.

THE STRUCTURE AND BIOLOGICAL FUNCTION OF RIBONUCLEASE H

Ribonuclease H (RNase H) plays crucial roles in replication of retroviruses, including the human immunodeficiency virus (HIV), and is involed in a variety of normal cellular events including DNA replication. The purpose of the project conducted by ***Robert Crouch*** and his colleagues is to gain a detailed understanding of the nature of the substrates for RNases H and the chemical makeup of cellular RNases H.

Structural and functional comparison between retroviral and cellular RNases H. Ribonuclease H of reverse transcriptase and cellular RNases H are related proteins, both structurally and enzymatically. However, they differ in several aspects. Experiments designed to determine the sequences and structures that contribute to the differences between the two types of RNases H have been a focus of this project. Crouch and colleagues have expressed the RNase H domains of HIV and MuLV as separate proteins and studied some of its properties related to binding to RNA-DNA hybrids. *E. coli* RNase HI is more than 1,000 times as active enzymatically as either of the RNases H derived from the mammalian retroviruses. *E. coli* RNase HI binds very rapidly to RNA-DNA hybrids as does the MuLV RNase H. Thus, the differences in specific activity between these two enzymes is due to a difference in catalytic activity. In contrast, an *E. coli* RNase H protein deleted for the region known to be responsible interacting with RNA-DNA substrates (making it similar to HIV RNase H) binds very poorly to its substrate.

Targeting of *E. coli* RNase H to retroviruses. The different activities of viral and bacterial RNases H suggest that if one were to replace the viral with the bacterial enzyme within a retrovirus, severe consequences for its replication might result. When Crouch and colleagues targeted *E. coli* RNase H to mammalian retroviruses and yeast retrotransposons, the differences in enzymatic properties resulted in inhibition of viral multiplication in the case of MuLV, TY-1 and possibly HIV. Thus, targeting of cellular RNase H to virus particles may be a useful method for containing retroviral infections.

LABORATORY OF MOLECULAR GENETICS

Chief, Igor B. Dawid, Ph.D.

Principal Investigators
C. Michael Cashel, M.D., Ph.D.
Robert J. Crouch, Ph.D.
Judith G. Levin, Ph.D.
Thomas D. Sargent, Ph.D.
Robert A. Weisberg, Ph.D.

Tenure-Track Investigators
Susan R. Haynes, Ph.D.
James A. Kennison, Ph.D.

Senior Fellows
Susana Cerritelli, Ph.D.
Alexander Karavanov, Ph.D.
Maria I. Morasso, Ph.D.
Masanori Taira, Ph.D.
Reiko Toyama, Ph.D.

Post-Doctoral Fellows
Margaret Adam, Ph.D.
Arulvathani Arudchandran, Ph.D.
Sarbani Banik-Maiti, Ph.D.
Joseph Breen, Ph.D.
Jeffrey Franklin, Ph.D.
Eric Glasgow, Ph.D.
Jianhui Guo, M.D.
Li-ying Han, Ph.D.
Rodney King, Ph.D.
Wu-Po Ma, Ph.D.
Nino Mzhavia, Ph.D.
Catherine Nocente-McGrath, Ph.D.
Cecilia Pazman, Ph.D.
Michael Powell, Ph.D.
Michael Rebagliati, Ph.D.
Jean-Pierre Saint-Jeannet, Ph.D.
Jon Scales, Ph.D.
Samuel Stoler, Ph.D.
David Stolow, Ph.D.
Daniel Vinella, Ph.D.
Hongtao Wu, Ph.D.
Tiyun Wu, Ph.D.
Wei-Xing Wu, M.D., Ph.D.

Hirofumi Yamada, Ph.D.
Xinyi Zhan, Ph.D.

Pre-Doctoral Fellows
Rosemary Wu
Kevin Eggan

Guest Researchers
Naina Bhatia-Dey, Ph.D.
Ya-Xiong Feng, Ph.D.
Xi He, Ph.D.
Victor Hernandez, Ph.D.
Tetsuhiro Kudoh, Ph.D.
Ezra Yagil, Ph.D.

Senior Technical Specialists
Helen Murphy
Klara Post
Martha Rebbert
Sieghild Sloan

LMG - FY96 Annual Bibliography

Appel V, Korzh V, Glasgow E, Thor S, Edlund T, Dawid IB, Eisen JS. Motoneuron fate and patterned LIM homeobox gene expression in embryonic zebrafish. Development 1995;121:4117-25.

Cashel M, Gentry DR, Hernandez VJ, Vinella D. The Stringent Response. In Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology. (Neidhardt, FC, Curtiss III R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff W, Riley M, Schaecter M, and Umbarger AE, eds) 2nd Ed ASM Press Washington, DC, 1996;1458-96.

Dawes SS, Crouch RJ, Morris SL, Mizrahi V. Molecular cloning, sequence analysis, overproduction in *Escherichia coli* and enzymatic characterization of the ribonuclease HI from *Mycobacterium smegmatis*, Gene 1995;165:71-75.

Dorgai L, Yagil E, Weisberg RA. Identifying determinants of recombination specificity: Construction and characterization of mutant bacteriophage integrases. J Mol Biol 1995;252:178-88.

Feng YX, Copeland TD, Henderson LE, Gorelick RJ, Bosche WJ, Levin JG, Rein A. HIV-1 nucleocapsid protein induces “maturation” of dimeric retroviral RNA in vitro, Proc Natl Acad Sci USA 1996;93:7577-81.

Franklin JL, Sargent TD. VN-Cad, a novel cadherin expressed in a subset of neural tissues in the zebrafish embryo. Dev Dynamics 1996;206:121-30.

Gentry DR, Cashel M. Mutational analysis of the Escherichia coli spoT gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation. Mol Microbiol 1996;19:1373-84.

Haynes SR. Posttranscriptional regulation and RNA binding proteins in development. J Biomed Sci 1995;2:293-301.

Hernandez VJ, Hsu LM, Cashel M. Conserved region 3 of Escherichi coli sigma-70 is implicated in the process of abortive transcription. J Biol Chem 1996;271:18775-18779.

Hernandez VJ, Cashel M. Changes in conserved region 3 of Escherichia coli σ^{70} mediate ppGpp-dependent functions in vivo. J Mol Biol 1995;252:536-49.

Karavanov AA, Saint-Jeannet J-P, Karavanova I, Taira M, Dawid IB. The LIM homeodomain protein Lim-1 is widely expressed in neural, neural crest and mesoderm derivatives in vertebrate development. Int J Dev Biol, 1996;40:453-61.

Kennison JA. The Polycomb and trithorax group genes of Drosophila: Trans-regulators of homeotic gene function, Annual Reviews of Genetics 1995;29:289-303.

Ma W-P, Crouch RJ. *Escherichia coli* RNase HI inhibits MuLV reverse transcription in vitro and TY1 retrotransposition in vivo, Genes to Cells 1996;1:581-593.

Mechold U, Cashel M, Steiner K, Gentry DR, Malke H. Functional analysis of a relA/spoT homolog from Streptococcus equisimilis. J Bacteriol 1996;178:1401-11.

Minucci S, Saint-Jeannet J-P, Toyama R, Scita G, DeLuca LM, Taira M, Levin AA, Ozato K, Dawid IB. Retinoid X receptor-selective ligands produce malformations in *Xenopus* embryos. Proc Nat Acad Sci, 1996;93:1803-7.

O'Connell ML, Rebbert ML, Dawid IB. Isolation of the *Xenopus nrp-1* gene and analysis of its expression following the midblastula transition in injected *Xenopus* embryos. Develop. Growth Differ., in press.

Powell MD, Levin JG. Sequence and structural determinants required for priming of plus-strand DNA synthesis by the human immunodeficiency virus type 1 polypurine tract, J Virol 1996;70:5288-96.

Scales JB, Winning RS, Renaud CS, Shea LJ, Sargent TD. Novel members of the Eph receptor tyrosine kinase subfamily expressed during Xenopus development. Oncogene 1995;11:1745-52.

Taira M, Evrard J-L, Steinmetz A, Dawid IB. Classification of LIM proteins. Trends Genet 1995;11:431-2.

Toyama R, Curtiss P, Otani H, Kimura M, Dawid IB, Taira M. The LIM class homeobox gene *lim5*: implied role in CNS patterning in *Xenopus* and Zebrafish. Dev Biol 1995;170:583-93.

Vinella D, Gagny B, Joseleau-Petit D, D'Ari R, Cashel M. Mecillinam resistance in Escherichia coli is conferred by loss of a second activity of the aroK protein. J Bacteriol 1996;178:3818-26.

Weisberg RA, Freundlich M, Friedman DI, Gardner JF, Goosen N, Nash HA, Oppenheim A, Rouviére-Yaniv J. Nomenclature of the genes encoding IHF. Mol Microbiol 1996;19:642.

Weisberg RA. Specialized transduction. In: *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, edited by Neidhardt FC, Curtiss RC III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE. Washington DC. ASM Press 1996, p 2442-2448.

Winning RS, Scales JB, Sargent TD. Disruption of cell adhesion in Xenopus embryos by kinase activity of Pagliaccio, and Eph-class receptor tyrosine kinase. Dev Biol In Press.

Wu W, Henderson LE, Copeland TD, Gorelick RJ, Bosche WJ, Rein A, Levin JG. Human immunodeficiency virus type 1 nucleocapsid protein reduces reverse transcriptase pausing at a secondary structure near the murine leukemia virus polypurine tract, J Virol, in press.

Yagil E, Dorgai L, Weisberg RA. Identifying determinants of recombination specificity: Construction and characterization of chimeric bacteriophage integrases. J Mol Biol 1995;252:163-77.

Zu K, Sikes ML, Haynes SR, Beyer AL. Altered levels of the *Drosophila* HRB87F/hrp36 hnRNP protein have limited effects on alternative splicing in vivo. Molec Biol Cell 1996;7:1059-73.

Project Numbers - Annual Report 1996

LABORATORY OF MOLECULAR GENETICS (LMG)

ZO1 HD 00066-26	Control Mechanisms in Temperate Bacteriophage Lambda Robert A. Weisberg, Ph.D.
ZO1 HD 00067-28	The Integration of Macromolecular Synthesis in Escherichia coli Michael Cashel, M.D., Ph.D.
ZO1 HD 00068-25	Factors Influencing Genetics Transcription-Initiation and Termination Robert J. Crouch, Ph.D.
ZO1 HD 00069-24	Molecular Genetics of Mammalian Retrovirus Replication Judith G. Levin, Ph.D.
ZO1 HD 01002-14	Gene Expression During Embryonic Development of Xenopus Laevis Igor B. Dawid, Ph.D.
ZO1 HD 01005-09	Regulation of Cellular Proliferation and Diversity in Drosophila James A. Kennison, Ph.D.
ZO1 HD 01006-08	Protein-Nucleic Acid Interactions in Vertebrate Embryogenesis Thomas D. Sargent, Ph.D.
Z01 HD 01008-07	Molecular Genetics of Protein-Nucleic Acid Interactions in Drosophila Susan R. Haynes, Ph.D.

Z01 HD 01011-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00066-26 LMG

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control Mechanisms in Temperate Bacteriophage λ

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert A. Weisberg	Section Head	LMG:NICHD
Others:	Banik-Maiti, Sarbani	Visiting Fellow	LMG:NICHD
	Rodney King	NRC Fellow	LMG:NICHD
	Nino Mzhavia	Visiting Fellow	LMG:NICHD
	Sloan, Sieghild	Microbiologist	LMG:NICHD
	Jennie Tang	Summer IRTA	LMG:NICHD

COOPERATING UNITS *(if any)*

Department of Biochemistry, Tel Aviv University, Israel (Ezra Yagil); Laboratory of Molecular Biology, NCI (Ding Jun Jin)

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.2	4.0	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Antitermination increases the transcription of genes that are located downstream of terminators. Transcripts encoded by the cis-acting antitermination sites (*put* sites) of lambdoid phage HK022 promote readthrough of downstream transcription terminators. Proper conformation of the transcripts is essential for activity, since *put* mutations that prevent the formation of predicted RNA stems prevented antitermination, and suppressor mutations that restore the stems restored antitermination. Antitermination does not appear to require proteins other than RNA polymerase, since *put*-dependent readthrough of multiple sequential terminators was observed in a purified transcription system containing only template, polymerase, substrates, buffer, and product. Transcription of *put* also increased the elongation rate of polymerase, very likely by suppressing pausing. A mutation that alters the zinc-finger region of the β′ subunit of polymerase specifically prevented the *put*-dependent increases in terminator readthrough and elongation rate. The simplicity of HK022 antitermination contrasts with that of other known antitermination pathways. We propose that the central effector is a transcript that directly alters the elongation properties of RNA polymerase. The integrase proteins of phages λ and HK022 are closely related site-specific recombinases that recognize different nucleotide sequences in the core regions of their substrates, the attachment sites of the two phages. We have now identified several attachment site nucleotides that inhibit recombination by the non-cognate integrase. These include G4 in the λ B′ core site and A1 and C3 in the HK022 B′ and C core sites. Interestingly, nucleotides flanking the canonical core sites also appear to be recognized by integrase.

Project Description:

Objective: To understand the mechanisms of genetic recombination, especially as exemplified by the insertion of the bacteriophage λ genome into the *Escherichia coli* chromosome, and to understand how viruses control the expression of their genes.

Major Findings.

1. Structure of the HK022 *put* sites .

We have previously shown that HK022 antiterminates early transcription, but that it does so in a way that differs from and appears far simpler than previously characterized antitermination systems. The only specific requirements for antitermination appear to be cis-acting phage sites called *put*L and *put*R, located in the HK022 P_L and P_R operons, respectively, and a protein domain (the "zinc-binding region") located near the amino-terminal end of the β′ subunit of *E. coli* RNA polymerase. The many protein factors needed in other antitermination systems are not required by HK022. In order to define the *put* sites and to ask if they interact with the zinc-binding region of β′, we have developed a genetic screen to isolate and to characterize mutants that alter antitermination (see last year's annual report). Using this system, we showed that *put*L is located within the first 76 bp of the P_L operon, and that *put*R is located between positions 275 and 341 of the P_R operon (Figure 1). These two sites are similar to each other, and their transcripts can be folded into two stem-loops separated by a 1 nt spacer. Several base-pairs that are conserved in the two folded structures occur at nonconserved positions (indicated by "☞" in Figure 1), suggesting that the two the *put* sites act as RNA. This suggestion is strongly supported by the phenotypes of mutations of stem nucleotides: in every case tested, changes predicted to destabilize either of the stems was reduced for antitermination and suppressor mutations predicted to restore the stems restored antitermination.

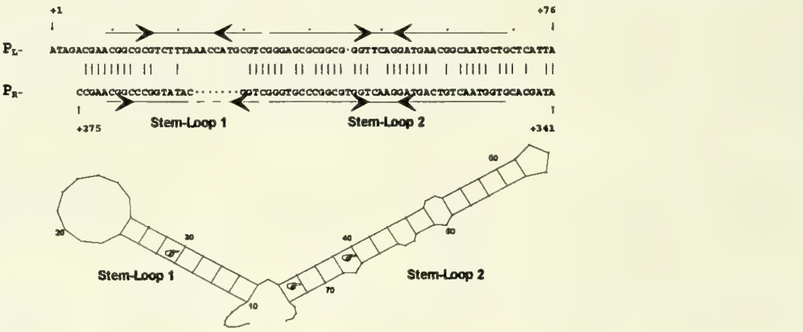

+1
+76
P_L-
ATAGACGAACGGCGCGTCTTTAAACCATGCGTCGGGAGCGCGGCG·GGTTCAGGATGAACGGCAATGCTGCTCATTA
P_R-
CCGAACGGCCCGGTATAC······GGTCGGGTGCCCGGCGTGGTCAAGGATGACTGTCAATGGTGCACGATA
+275
Stem-Loop 1
Stem-Loop 2
+341
60
20
30
40
50
70
10
Stem-Loop 1
Stem-Loop 2

Figure 1. **Top:** The *put*L (first line) and *put*R (second line) sites of HK022 are aligned so as to maximize sequence and secondary structure similarities. The numbers +1 and +275 indicate position relative to the starts of the P_L and P_R transcripts, respectively. **Bottom:** Base-pairings in the predicted *put*L structure are shown. "☞" marks base-pairs that vary between *put*L and *put*R in such a way as to preserve pairing.

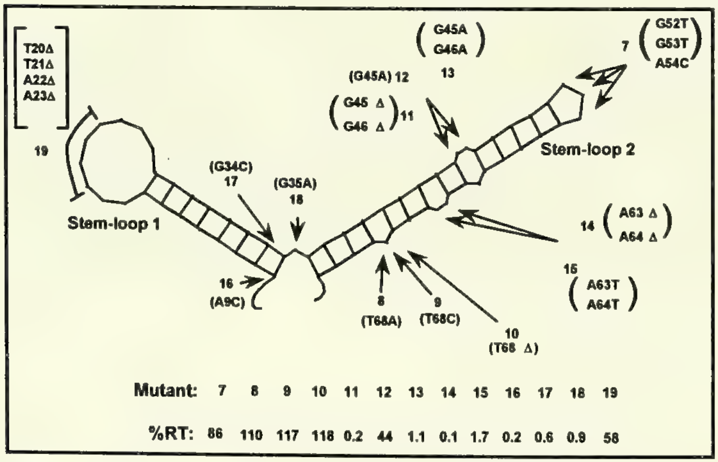


Figure 2. The effects of changing unpaired positions of *put*L. Per cent readthrough ("%RT") approximately 100% for wild type *put*L.

More recently, we have probed the structure of *put*L and *put*R RNAs by determining the pattern of sensitivity to nucleases that prefer to attack single or double stranded RNA. The results of this study were, in general, confirmatory of the computer prediction shown in Figure 1. One exception to this rule was the bases in the internal loop in the ascending part of stem 2 (positions 45 and 46), which are less susceptible than expected to single strand-specific nucleases, and are presumably involved in some unconventional type of base pairing or stacking. We also determined the phenotypes of mutations that change non base-paired positions of *put*L (Figure 2). It appears that the size and the identity of the nucleotides in loop 1 are unimportant for function. Most of this region is not conserved between *put*L and *put*R. The identity of the nucleotides in loop 2 are likewise unimportant, although they are conserved between the two *put* sites. The same is true for the bulge in the descending portion of stem 2. The identity of the base of the interstem spacer is important (although we have found that T is as functional as G), and so is that of the nucleotides of the internal loop of stem 2.

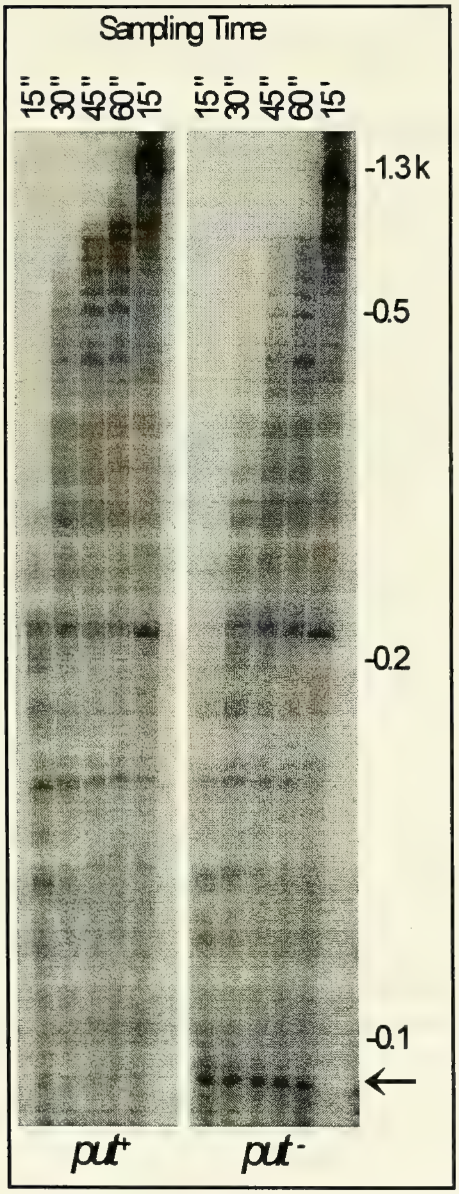


Figure 3. Kinetics of transcription of *put*$^+$ and *put*$^-$ templates by purified RNA polymerase. Initiation complexes were preformed, and transcription was initiated by addition of substrates. Transcript sizes are indicated in kb along the right margin.

2. The HK022 *put* sites accelerate RNA polymerase through pause sites

We have shown that the wild type *put*L site suppresses termination by purified RNA polymerase, and that suppression is prevented if polymerase is mutant in any one of several residues located within a zinc-binding domain of the β′ subunit. This suggests that antitermination requires a specific interaction between *put* RNA and the zinc-binding domain of polymerase. It is known that RNA polymerase does not transcribe DNA at a uniform rate. Various characteristics of the template induce pauses that can endure for seconds to minutes according to the site, and there is much evidence that correlates pausing and termination. To see if *put* influences elongation rate, we measured the time required for synchronized populations of RNA polymerase molecules to reach the ends of two linear templates that lack strong terminators and differ by a *put*L base substitution mutation. Figure 3 shows a representative electropherogram. We estimated the speed of the fastest polymerase molecules by linear extrapolation of the rate of accumulation of run-off transcripts to zero accumulation. We found that run-off transcripts begin to accumulate at a linear rate by 15 sec after initiation for the wild type template and 20 sec for the mutant. The corresponding elongation rates were 39 nt/sec and 27 nt/sec, respectively. A template with a different *put* mutation (linker scanning mutant G) gave results that were similar to those obtained with the first mutant (data not shown). By contrast, polymerase containing the RpoC-Y75N mutant subunit transcribed both the *put*L$^+$ and the *put*L$^-$ template at rate of 24 nt/sec (data not shown). We conclude that *put*L increases the rate of elongation, and that the requirements for this increase are the same as those for antitermination. It is highly likely that the increased elongation rate reflects acceleration of polymerase through some of the pause sites that can be seen in Figure 3. For example a cluster of transcripts about 85-95 nt long appeared only when the template was *put*⁻ or polymerase contained the RpoC-Y75N mutant subunit (arrowhead; data not shown for mutant polymerase). These transcripts reached a level of about 30 to 50% of the total soon after transcription was initiated, and either gradually decreased after 15 min (mutant polymerase) or remained approximately constant (*put*⁻ template). A substantial fraction of the 85-95 nt transcripts produced on a *put*⁻ template remained associated with the transcription complex during ultrafiltration of the reaction, and are therefore the result of polymerase arrest rather than termination (data not shown). Since this arrest site is located immediately downstream of *put* and because arrest is suppressed by a wild type *put* site, we infer that *put* RNA interacts with polymerase very shortly after it is synthesized. Arrest at this site can be eliminated without interfering with *put* antitermination function by a deletion that removes DNA downstream of position 76, so it appears that polymerase arrest at this site is not a requirement for antitermination.

3. Identification of nucleotides that are differentially recognized by HK022 and λ integrases.

Integrases catalyze recombination between DNA molecules that carry specific sequences called attachment sites. The HK022 and λ integrases are two very closely related proteins (70% identity) that recognize different sequences within the core regions of their respective attachment sites. We have previously identified the integrase residues that are responsible for the specificity difference between the two proteins (see last year's Annual Report). However, comparison of the DNA sequences believed to be differentially recognized by the two integrases does not suggest which nucleotides are integrase-specific. To identify these nucleotides, we constructed and characterized a large number of recombination reporter substrates that contain a pair of attachment sites with core region mutations. The phage and bacterial attachment sites of HK022 and λ have 4 core sites (called B, B', C, and C'), and each differs from the others in nucleotide sequence. Figure 4 shows the "canonical" core sites of the two phages together with flanking DNA. We have now shown that G at position 4 of the λ B' core site impedes recombination by HK022 integrase, and A and/or C, at positions 1 and 3, respectively, of the HK022 C and B' core sites impede recombination by λ integrase. In addition, we found that nucleotides immediately adjacent to the

	λ <-FLANKS-><---CORE SITES--->	HK022 <-FLANKS-><---CORE SITES--->
Position:	-3 -2 -1 1 2 3 4 5 6 7↓8 9	-3 -2 -1 1 2 3 4 5 6 7↓8 9
Site B:	5'- A G C C T G C T T T T T	5'- G G T G C A C T T T A G
Site B′:	5'- G C T C A A G T T A G T	5'- C T C A A C C T T T T T
Site C:	5'- G T T C A G C T T T T T	5'- G T C A T C C T T T A G
Site C′:	5'- T G C C A A C T T A G T	5'- A T A C A A C T T A T T

Figure 4. Sequences of the λ and HK022 core sites and the flanking regions. The shaded bases are likely to be determinants of recombination specificity (see text). These sites were originally identified in λ by sequence comparisons and by footprinting with Int. B and B' are in the bacterial attachment sites, and C and C' are within the phage attachment sites. The two core sites are arranged as pair of imperfect inverted repeats separated by a spacer sequence, and DNA strand exchange occurs at the positions indicated by the vertical arrows.

canonical core sites are important negative determinants of specificity for HK022 integrase. This includes the T residues at position -1 flanking the C and B' sites (Figure 4). This is a surprising result because the flanking bases were not known to be recognized by Int, and because wild type HK022 has a T in position -1 flanking the B site. It appears that functional divergence between the λ and HK022 attachment sites has occurred by acquisition of nucleotides that are unacceptable to the non-cognate integrase.

Proposed Course of Project:

1. Antitermination of early transcription in HK022. We plan to continue our characterization of the HK022 *put* sites by constructing and analyzing mutants as described above. We will also attempt to select compensatory mutations that either suppress the effect of mutations in unpaired bases or suppress the effect of mutations in the β′ subunit of RNA polymerase. We also plan to analyze the zinc binding region of the RNA polymerase β′ subunit by alanine scanning mutagenesis. We will screen for haplo-lethal, dominant lethal, and antitermination-defective mutants. We will also ask if any mutations in this region can suppress antitermination-defective *put* mutants, and, if so, if they are mutation-specific. The same selective system is also suitable for isolating antitermination-resistant transcription terminators. If such terminators exist, we will analyze their function.

2. Antitermination with purified proteins. (*i*) We plan to test newly isolated *put* and RNA polymerase mutants *in vitro* to confirm that antitermination with purified RNA polymerase faithfully reflects antitermination within cells. The efficiency of terminator readthrough *in vitro* appears to be about one-half of what it is *in vivo*. We will verify this conclusion by using a more direct assay for antitermination *in vivo*. If it is confirmed, we will search for host proteins that increase antitermination efficiency. (*ii*) Persistent antitermination is likely to involve a stable interaction of the *put* transcript with polymerase. We will look for such an interaction in two ways. First, we will attempt to cross-link *put* RNA to polymerase using photo-activated cross-linkable base analogues that are incorporated into specific positions of the transcript. If we find *put*-dependent cross-linking, we will map the position of the cross-link by controlled protease digestion of the polymerase subunit that is cross-linked. Second, we will prepare synchronized ternary transcription complexes that are paused at particular positions on the template and look for RNA loops with the electron microscope. A stable interaction between *put* and polymerase should produce RNA loops whose sizes depend on the position of the paused complex. Third, we will purify *put* RNA and look for evidence of a interaction with RNA polymerase by sequence-specific interference with or promotion of terminator readthrough, gel retardation and filter binding. If any of these techniques gives positive results, we will attempt to identify the region of polymerase that is bound to RNA by means of RNA-protein cross-linking and protein footprinting experiments.

3. Recognition of attachment sites by integrase proteins. (*i*) Binding of integrase to core binding sites is weak and difficult to detect under recombination conditions. However, if a DNA molecule that contains a core binding site also has an arm binding site, simultaneous binding of integrase to both sites promotes bending of the DNA, and such bending can be detected by binding of a second protein, such as HU, that has affinity for bent DNA. We plan to use this technique to ask if integrase mutations that switch or relax the specificity of recombination also alter binding to core sites of different specificities. (*ii*) Now that we have identified the nucleotides that confer integrase-specificity on attachment

sites, we will ask if the recombination-inhibiting effect of particular nucleotide changes can be suppressed in an allele-specific fashion by particular amino acid changes in integrase. If so, this will provide evidence about residue-specific Int-attachment site interactions.

Significance to Biomedical Research and the Program of the Institute:

The insertion of viral chromosomes into host chromosomes changes the physiology of the host organism. For example, temperate bacteriophages carry genes that can render the intermediate bacterial host harmful to an animal or human host. In addition, viruses provide an important mechanism of genetic transfer and exchange in the microbial world. Viruses promote genetic transfer because of their ability to convert host genes to an infectious form by packaging them inside a shell of viral structural proteins. The successful symbiotic relationship between temperate bacteriophages and bacteria requires intermeshing between the life cycles of virus and host at many different points. An understanding of the control mechanisms operative in these easily studied microorganisms has obvious relevance to an understanding of regulatory elements in higher organisms.

Publications:

Dorgai, L., Yagil, E., and Weisberg, R.A. Identifying determinants of recombination specificity: Construction and characterization of mutant bacteriophage integrases. *J.Mol.Biol.* 252:178-188, 1995.

Weisberg, R.A. Specialized transduction. In: *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, edited by Neidhardt, F.C., Curtiss, R.C.,III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M., and Umbarger, H.E.Washington, DC:ASM Press, 1996,p. 2442-2448.

Weisberg, R.A., Freundlich, M., Friedman, D.I., Gardner, J.F., Goosen, N., Nash, H.A., Oppenheim, A., and Rouvière-Yaniv, J. Nomenclature of the genes encoding IHF. *Mol.Microbiol.* 19:642, 1996.

Yagil, E., Dorgai, L., and Weisberg, R.A. Identifying determinants of recombination specificity: Construction and characterization of chimeric bacteriophage integrases. *J.Mol.Biol.* 252:163-177, 1995.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00067-28 LMG

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Integration of Macromolecular Synthesis in Escherichia coli

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Cashel, MD PhD	Section Chief	LMG:NICHD
Others:	D. Gentry, PhD	IRTA	LMG:NICHD
	D. Vinella PhD	Visiting Fellow	LMG:NICHD
	V. J. Hernandez, PhD	IRTA	LMG:NICHD
	H. Murphy	Chemist, Lab. Tech.	LMG:NICHD

COOPERATING UNITS *(if any)*

Dr. Gad Glaser, Hadassah Med. School, Jerusalem, Israel; Dr. Arthur Kornberg, Stanford, CA; Dr. Horst Malke, Jena, Germany; Dr. Richard D'Ari, Paris, France.

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Section on Molecular Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.2	3.2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The expression of the genomic repertoire of Escherichia coli is coordinately regulated in response to nutrient availability. This determines growth as well as the ability to survive prolonged periods of starvation. This project focuses on regulation of these processes in bacteria by analogs of the common nucleotides GDP and GTP that possess ribosyl 3' pyrophosphate esters. This family of compounds is abbreviated collectively as (p)ppGpp. Intracellular levels of (p)ppGpp are altered in response to availability of amino acids, phosphate, nitrogen or energy; the (p)ppGpp signal, in turn, triggers globally altered patterns of gene expression related to the specific nutritional stress. In addition, (p)ppGpp recruits global domains of gene expression associated with stationary phase. This year we have found evidence of recruitment of cell cycle regulation domains and gene domains responding to the leucine regulatory protein (lrp) gene. Current evidence arising from mutant analyses is therefore beginning to define the extent of global regulatory domains directly or indirectly regulated by (p)ppGpp. So far, mutants that phenotypically mimic the presence of (p)ppGpp, when this nucleotide is actually absent, are found to occur only in three RNA polymerase subunit genes. We have previously described two "housekeeping" sigma mutants and their effects on premature RNA chain release during transcription initiation on model promoters. This year these effects are extended to suggest that physiologically relevant promoters responsible for synthesis of galactose or histidine are stimulated by the mutants by increasing promoter clearance efficiency. Attention is now given to other RNA polymerase mutants. We have mapped and identified the sequence changes for 59 different alleles of genes encoding the two large "core" RNA polymerase subunits (beta and beta'). In all, 52 new alleles have been documented. There is early evidence that these mutants do not regulate galactose promoters in the same way as previous sigma mutants. They are therefore of great interest.

Project Description

Objectives

We wish to understand how cellular gene expression is coordinated during adaptation to the stress of transitions between nutritional impoverishment and abundance. Nutritional stress is studied because it is arguably the most common biological stress. Escherichia coli is studied, with its full array of stress responses, because the molecular level of understanding of this organism is by far the most advanced of any organism capable of an independent existence. Although stress responses of other organisms are known to show mechanistic differences, operating biological principles are likely to be shared. The genetic repertoire of E. coli is organized into functional domains or "regulons" that are operationally defined by shared regulatory responses to a given stress. Expression of multiple regulons can also be linked, culminating in an integrated cellular response at the level of macromolecular synthesis, gene expression and growth. Our overall objective is to understand how this works, starting with one global regulatory signal, (p)ppGpp, and then dissecting out regulatory interconnections.

The stress signals and macromolecules considered in this project have broadened considerably since the time it was originally aimed at the regulation of stable RNA accumulation occasioned by the relA gene-mediated ppGpp accumulation when amino acids or aminoacyl-tRNA availability limited exponential growth. Currently, we can artificially manipulate ppGpp levels from complete absence to over-abundance without provoking nutrient changes. Mutants suppressing the consequences of these manipulations are readily isolated and characterized to provide clues to the regulatory targets involved. In this manner regulatory pathways can be discovered. The objectives are: 1) to learn of the stress systems that provoke (p)ppGpp accumulation; 2) to understand the mechanisms involved in perturbations of (p)ppGpp metabolism; and 3) to define the regulatory effects on gene expression and cell biology exerted by (p)ppGpp.

Methods Employed

Methods have been published.

Major Findings

1. Characterization of RNA polymerase mutant sigma-70 subunit suppressors of a (p)ppGpp-deficient phenotype. Regulatory effects of (p)ppGpp have long been thought to occur at the transcriptional level but there is evidence favoring both initiation and elongation as the steps involved. In previous annual reports, we focused attention on the rpoD mutants because mutant effects were likely to be limited to transcription initiation. We localized two mutant sites (P504L and S506F) to a region between protein domains that recognize the -35 and -10 promoter consensus elements. In vivo and in vitro studies (now published) reveal that the reconstituted mutant RNA polymerase reduces abortive RNA chain formation as a complex function of chain length when tested with three sigma-70-dependent "model" promoters. These promoters were chosen for study because they represented a quantitative spectrum of tendencies

to abortively release RNA chains during initiation. Although abortive RNA chain release was reduced by the reconstituted mutant enzymes, overall (full length) transcription activity measured with these model promoters did not increase. Analogous studies, about to be published, with the physiological gal operon promoters from E. coli have revealed that the pattern and abundance of abortive RNAs again was altered for the mutant enzymes. Importantly, the abundance of full length gal transcripts was increased collectively to the same extent as the decrease in abortive RNA chains, about 3- to 4-fold. Since the kinetic constants for promoter binding and for the rate of open DNA complex formation did not change, the sigma mutants are judged to increase promoter strength by increasing the efficiency of promoter clearance. In vivo assays of effects of the sigma mutants on gal-lacZ fusion activities in (p)ppGpp-0 hosts have revealed quantitatively equivalent (3-4X) regulatory effects on beta-galactosidase activity. These observations establish the long sought after congruence between in vivo and in vitro mutant behavior. However, a caveat is that addition of ppGpp to the galP2 transcription reactions with wild-type RNA polymerase did not alter full length product formation. An additional component may be missing from the highly purified reaction components.

To ask if the mutant ability to increase promoter clearance can be generalized, equivalent studies have been performed in vivo and in vitro with histidine operon promoters long known to be activated by ppGpp. Initial indications are that regulatory behavior for the his promoters is qualitatively similar to gal but quantitatively more dramatic with 6-8X effects. Thus, the sigma-70 mutants may generally activate ppGpp-inducible promoters by reducing the frequency of abortive RNA chains during initiation and increasing promoter clearance. It is still unproven whether ppGpp has the same mechanism of action with wild type RNA polymerase.

2. Characterization of RNA polymerase mutant beta (rpoB) and beta' (rpoC) subunit suppressors of a (p)ppGpp-deficient phenotype. Analysis of suppressor mutants not mapping in rpoD (above) has led to their localization in the rpoB and rpoC genes. Thus, mutants acting as suppressors of a (p)ppGpp-deficient phenotype are so far found exclusively in RNA polymerase genes. We initially thought that these mutations would behave like the sigma mutants just described, but this is not observed. All RNA polymerase core mutants tested so far apparently lack the ability to activate gal promoter activity. This unpredicted behavior is interesting. The mutants might alter (p)ppGpp binding since a ppGpp site has been reported to exist on core RNA polymerase. Convincing accounts of (p)ppGpp on elongational pausing have also appeared in the literature; correspondingly, the core subunit mutants might be imagined as involving this process as an alternative to transcription initiation

Several hundred mutations were first mapped by phage P1 transduction, then mapped to rpoB or rpoC by plasmid complementation studies, then mapped within the 8 Kbp rpoBC region by sensitization of RNA-RNA (wildtype-mutant) heteroduplex mismatches to RNase digestion and finally sequenced with synthetic primers. The results of this effort are that 34 new alleles in rpoB have been defined; 6 were known to exist from our previous work. The rpoC gene was found as the site of 18 new alleles with only one known previously. Mutant sites are clustered in specific regions of rpoB and rpoC; since functional domains are currently partially defined for these genes, the clusters provide clues to their possible function. So far, clues are not answers; there is no simple explanation to the observed pattern of mutant location. More than one mechanism may be possible or a new function is awaiting definition.

We are beginning to sort the 59 mutants into functional classes, based on suppression strengths and other criteria, such as the ability to generate resistance to the antibiotic mecillinam (see below). The goal is to ultimately identify the target promoters affected by the RNA polymerase mutants and study mutant effects on their activities. There is evidence from other laboratories that ppGpp serves to induce the leucine regulatory protein (abbreviated as Lrp) which, in turn, can have complex regulatory effects on amino acid metabolism. In cells lacking ppGpp, many of the lesions in amino acid metabolism can be explained as due to the inability to express Lrp; mutants that correct these metabolic lesions could work by increasing Lrp expression. Indeed, we have found that transducing a lrp insertion mutation into some (not all) mutants reverses the suppressed prototrophic state and restores the auxotrophy for a nearly complete spectrum of amino acids found the absence of ppGpp. This indicates that Lrp function is induced by the these mutations. The regulatory target could be either the lrp promoter or some element required for Lrp function. Western blots with Anti-Lrp indicate that Lrp protein does not change by more than a factor of two at most, supporting the latter possibility. Other rpoBC mutants, in the presence of the lrp insertion, show few amino acid requirements or none. This behavior suggests the opposite conclusion, namely that suppression does not involve Lrp function. The fact that both sorts of mutants arise from the same selection procedure implies the existence of multiple targets of suppression.

3. Mecillinam resistance as a positive selection for new mutants affecting ppGpp and targets of ppGpp regulation. Dr. Richard D'Ari and colleagues (Paris) have found that mecillinam, a beta-lactam antibiotic that specifically inactivates penicillin binding protein 2 (PBP2) and results in cell death associated with inhibition of cell division. Cells become spherical in the presence of the antibiotic. This shape change is viewed as increasing the demand for FtsZ protein because viability is restored by overexpression of the ftsZ gene. The Fts protein is a tubulin-like protein whose polymerization is an early step in the formation of the septum between dividing cells. Studies in Paris have led to concluding that ppGpp accumulation mediated by the relA gene confers a mecR phenotype and ppGpp is possibly involved in cell cycle regulation. These studies led us to realize that selections for mecR in a relA deleted host could lead to the identification of new genes involved in regulation of ppGpp, FtsZ and cell cycle functions.

A search for new mecR mutants in a relA1 mutant host was initiated in Paris by Dr. Daniel Vinella using random transpositions with a miniTn10::kan element. Dr. Vinella came to NIH with an isolate obtained from this search and characterized the phenotype as due to a second function of AroK co-existing with its known shikimate kinase activity. We suspect this function is a protein kinase. Dr. Vinella has now sequenced ten insertions that confer the mecR phenotype to a relA deleted host. Some of these insertions increase ppGpp levels and these revert to mecS when ppGpp is abolished by deleting the spoT gene. Two mecR mutants do not show elevated ppGpp levels, when the spoT gene is present, but nevertheless revert to mecS when spoT is deleted. This mutant behavior suggests that the SpoT protein has another function besides those known involving ppGpp metabolism. Finally, three mutants remain mecR in the complete absence of ppGpp, clearly indicating a mechanism independent of ppGpp that possibly involves FtsZ recruitment or elevated specific activities for steps in FtsZ polymerization.

Since ppGpp confers a mecR phenotype and RNA polymerase mutants can mimic positive ppGpp regulation, we have also tested RNA polymerase mutants to see if they become mecR. In general, it has been found that the stronger mutants do give a mecR phenotype which underscores the idea that mecillinam resistance probably reflects a positive control function of ppGpp intimately related to cell cycle regulation. Unlike amino acid requirements, when these mutants are examined in a host also mutant for lrp, mecillinam resistance is unaffected. This implies that the target for ppGpp activation of the mecR phenotype is different from those for amino acid synthesis because it is not activated by Lrp.

Publications

Cashel M, Gentry DR, Hernandez VJ, Vinella D. The Stringent Response. In Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology. (Neidhardt, FC, Curtiss III R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff W, Riley M, Schaecter M, and Umbarger AE, eds) 2nd Ed ASM Press Washington, DC, 1996;1458-96.

Gentry DR, Cashel M. Mutational analysis of the Escherichia coli spoT gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation. Mol Microbiol 1996;19:1373-84.

Hernandez VJ, Cashel M. Changes in conserved region 3 of Escherichia coli σ^{70} mediate ppGpp-dependent functions in vivo. J Mol Biol 1995;252:536-49.

Hernandez VJ, Hsu LM, Cashel M. Conserved region 3 of Escherichi coli sigma-70 is implicated in the process of abortive transcription. J Biol Chem 1996;271:18775-18779.

Mechold U, Cashel M, Steiner K, Gentry DR, Malke H. Functional analysis of a relA/spoT homolog from Streptococcus equisimilis. J Bacteriol 1996;178:1401-11.

Vinella D, Gagny B, Joseleau-Petit D, D'Ari R, Cashel M. Mecillinam resistance in Escherichia coli is conferred by loss of a second activity of the aroK protein. J Bacteriol 1996;178:3818-26.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00068-25 LMG

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Factors Influencing Genetics Transcription-Initiation and Termination

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert J. Crouch, Ph.D.	Senior Investigator	LMG:NICHD
Others:	Susana Cerritelli	Senior Staff Fellow	LMG:NICHD
	Wu-Po Ma	Visiting Fellow	LMG:NICHD
	Hirofumi Yamada	Visiting Fellow	LMG:NICHD
	Li-Ying Han	Visiting Fellow	LMG:NICHD
	Makoto Wakasa	Guest Worker	LMG:NICHD
	Arulvanthani Arudchandran	Visiting Fellow	LMG:NICHD
	Kevin Eggan	Pre-IRTA	LMG:NICHD

COOPERATING UNITS *(if any)*

Section on Viral Gene Regulation, LMG:NICHD
Dr. Valerie Mizrahi, Molecular Biology Unit, The South African Institute for Medical Research, Johannesburg

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Section on Formation of RNA

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.7	4.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ribonuclease H of reverse transcriptase and cellular RNases H are related proteins, both structurally and enzymatically. However, they differ in several aspects. Experiments designed to determine the sequences and structures that contribute to the differences between the two types of RNases H have been a focus of some of our project. We have expressed the RNase H domains of HIV and MuLV as separate proteins and studied some of its properties related to binding to RNA-DNA hybrids. *E. coli* RNase HI is more than 1,000 times as active enzymatically as either of the RNases H derived from the mammalian retroviruses. *E. coli* RNase HI binds very rapidly to RNA-DNA hybrids as does the MuLV RNase H. Thus, the differences in specific activity between these two enzymes is due to a difference in catalytic activity. In contrast, an *E. coli* RNase H protein deleted for the region known to be responsible interacting with RNA-DNA substrates (making it similar to HIV RNase H) binds very poorly to its substrate. When we target *E. coli* RNase H to mammalian retroviruses and yeast retrotransposons, differences in enzymatic properties result in inhibition of viral multiplication for MuLV, TY-1 and possibly HIV. Thus, targeting of cellular RNase H to virus particles may be a useful method for containing retroviral infections.

Project Description:

Objectives:

The aim of this project is to examine the involvement of certain ribonucleases in cellular events. The scope of this project has evolved to include protein chemistry, enzymatic activity, overproduction of proteins and the consequences of changes in the level of enzymatic activity within the cell. In particular, ribonucleases specific for RNA of RNA-DNA hybrids are being examined for their functional role within the cell. Multiple enzymes exhibiting RNase H activity have been reported within cells and our experiments are aimed at understanding the need for multiple forms of the enzyme and what effects there would be upon inhibition of each (or all) of the RNase H activities. This is particularly relevant since the AIDS virus requires the functioning of its own RNase H to be infectious.

Major Findings:

Inhibition of retroviruses by *E. coli* RNase HI
Delivery of *E. coli* RNase HI to retroviral particles could be a valuable tool to (i) study the mechanism of retroviral replication and (ii) inhibit viral RNA replication and, thereby, limit the ability of these viruses to propagate. As a test system, we examined the TY-1 element of *Saccharomyces cerevisiae*, a retrotransposon that replicates as do retroviruses using a reverse transcriptase to make a DNA copy of an RNA genome. The DNA produced from TY-1 RNA can become inserted into a second location (a process called transposition). Virus-like particles are assembled from proteins encoded by the TY-1 RNA, including the capsid (or gag) protein. By constructing a gene fusing the coding sequence of the gag gene with that of the *E. coli* RNase HI gene, we can target RNase H to the viral particle and measure the effects on transposition. Inhibition of retrotransposition of more than 99% occurs when wild-type *E. coli* RNase HI is targeted to TY-1 particles but no detectable effect when a catalytically defective RNase HI is produced. These results suggested that replication of the RNA is the site of action of the *E. coli* enzyme. TY-1 and *S. cerevisiae* are very useful for genetic studies because of the power of selection of cells to examine. However, biochemical analysis is less tractable. We are now examining two mammalian retroviruses to determine whether they also are defective in replication of genomic RNA when *E. coli* RNase HI is present in the virus particle. Murine leukemia virus (MuLV) and human immunodeficiency virus (HIV) have been chosen for these studies. The MuLV project is a collaborative effort between our lab and that of Dr. Jef Boeke at Johns Hopkins University. Processing of the gag protein with *E. coli* RNase HI fused to it produces a pre-protein that becomes converted to a nucleocapsid-RNase HI protein fusion due to the MuLV protease (a normal event in processing pre-viral proteins to those observed in virus particles. The amount of fusion protein present in virus particles was determined by Western blot analysis and by liquid assays for RNase HI activity. Both determinations indicate significant levels of processed RNase HI with little evidence of unprocessed protein. This latter result is significant since it indicates that all of the fusion protein is accessible to the viral protease which can be derived only from MuLV virus. These virus particles are non-infectious (a decrease in infectivity to less than 10% that of wild-type

viruses. Thus, *E. coli* RNase HI can be effective in inhibition of mammalian retroviruses. Experiments using MuLV are performed by infection of cells that are transiently transfected with a plasmid bearing a transcriptional promoter to drive synthesis of the gag-*E.coli* RNase HI fusion protein. What would be necessary to produce cells in which a retrovirus would be inhibited by gag-*E. coli* RNase HI fusion proteins? First, production of *E.coli* RNase HI should not inhibit cell growth and second, gag-*E.coli* RNase HI fusion should not be inhibitory to cell growth. We have made stably transformed Jurkat cells which contain DNA sequences to produce *E. coli* RNase HI or gag-*E.coli* RNase HI fusion proteins. Neither type of cells creates a lethal situation for the cells. Infection of either of these cells with HIV-1 does not result in inhibition of viral replication as judged by production of p24, one of the virus proteins used to assess the presence of HIV viruses. Preliminary data measuring the RNA present in these virus particles suggests that at least one of the cell lines (two clonal cell lines from the same transfection) make viral particles with no viral RNA. If virus particles are made without packaging genomic RNA, they would still contain p24. HIV is a very complicated retrovirus and, unlike MuLV, requires a number of accessory proteins and RNAs for viral production. A sequence present in the HIV-1 genomic RNA called RRE for rev-responsive-element is necessary for transporting the RNA from the nucleus to the cytoplasm using the HIV encoded rev protein. Therefore, an RRE element on the RNA and rev protein are required for synthesis of the gag protein. The constructs we used for transforming the Jurkat cells contained no RRE element. Infection of these cells with HIV-1 would provide rev protein but RRE acts only in cis - not trans. It is possible that sequence differences DNAs used to genrate the various *E. coli* RNases HI clonal cell lines account for the differences in defects for HIV multiplication, possibly by permitting transport of the gag-*E.coli* RNase H message It would be almost ideal for therapeutic purposes if the gag-*E.coli* RNase HI fusion were not present in cells until infected with HIV.

Comparison of Binding Properties of Cellular and Viral RNase H to RNA-DNA Hybrids

E. coli RNase HI binds RNA-DNA hybrids through a structure known as the handle region and cleaves the RNA at a site involving three conserved carboxylic acid amino acids. HIV-1 RNase H does not have a handle region, but rather RNA-DNA binding occurs by binding of the hybrid to the polymerase domain. Expression of a protein containing only the RNase H domain of HIV-1 reverse transcriptase yields a protein that has no enzymatic activity. However, insertion of the handle region of *E. coli* RNase H at the corresponding location in the HIV-1 RNase H domain results in an enzymatically active RNase H protein. (See previous annual reports). MuLV reverse transcriptase has an RNase H domain that contains a handle region and when this domain is expressed as a separate protein, we find that it has enzymatic activity, similar to that observed when it is a part of the complete RT molecule (i.e., about 0.01% the specific activity of *E. coli* RNase HI).

Using surface plasmon resonance to measure the rates of binding and release of various RNases H, we conclude: (i) that *E. coli* RNase HI has a k_{on} rate of $1.5 \times 10^6\ M^{-1}s^{-1}$ and a k_{off} rate of $3.2 \times 10^{-2}\ s^{-1}$; and (ii) that using RNA-DNA hybrids where the RNA strand contains 2'-O-methylribose, these rates are k_{on} rate of $2.1 \times 10^5\ M^{-1}s^{-1}$ and a k_{off} rate of $1.3 \times 10^{-1}\ s^{-1}$. Since the 2'-O-methyl RNA-DNA hybrid is not cleaved by RNase HI, we determined these rates in the presence of Mg^{2+}. We found a reduction to a k_{on} rate of $7.4 \times 10^3\ M^{-1}s^{-1}$ and a k_{off}

rate of 2.6 × 10^{-2} s^{-1}. Thus, it appears that Mg^{2+} can affect the rates of binding of RNase HI, possibly through alteration is enzyme structure. We examined a mutant RNase H protein that is similar to HIV-1 RNase H (no handle region) and found its affinity for RNA-DNA hybrids to be 0.025% that of the wild-type protein. A point mutant changing Asp134 to Ala created an enzymatically inactive RNase H with binding characteristics similar to the wild-type protein. Thus, we can distinguish between inability to bind from lack of enzymatic activity. The MuLV RNase H domain has low activity due to poor catalytic cleavage not poor binding, as evidenced by kinetic binding and dissociation rates similar to wild-type *E. coli* RNase HI. This result suggests that other differences between these two RNases H account for the disparity in specific activities.

Multiple RNases H

In *E. coli* two RNases H have been identified; RNase HI, the enzyme with amino acid sequence and structural similarity to retroviral RNase H and RNase HII, an enzyme with little sequence homology to RNase HI. We have cloned a gene from *S. cerevisiae* with strong similarity to RNase HII of *E. coli* and have identified mouse and human clones obtained in genome sequencing projects which are related to RNase HII of *E. coli*. A search of the complete genome sequence of *S. cerevisiae* revealed no gene other than the RNase H1 gene we have described previously and will detail further below. However, from genomic DNA sequences of *Caenorhabditis elegans*, we find three RNase H genes related to *E. coli* RNase HI. One of them is a small gene encoding a protein about the same size as *E. coli* RNase HI, a second has a substantial C-terminal extension (an unique property) and a third that has a domain (perhaps two) with strong identity with the double-stranded RNA-binding domain present on RNases H1 of *S. cerevisiae*, *Schizosaccharomyces pombe* and *Crithidia fasciculata*. Thus, multiple RNases H exist in eukaryotic cells that are related to *E. coli* RNases HI and II.

Publications:

Journal Articles

Dawes SS, Crouch RJ, Morris SL, Mizrahi V. Molecular cloning, sequence analysis, overproduction in *Escherichia coli* and enzymatic characterization of the ribonuclease HI from *Mycobacterium smegmatis*, Gene 1995;165:71-75.

Ma W-P, Crouch RJ. *Escherichia coli* RNase HI inhibits MuLV reverse transcription in vitro and TY1 retrotransposition in vivo, Genes to Cells 1996;1:581-593.

Future Work:

I. Retroviral RNase H and retrovirus replication

A) Targeting Cellular RNases H to Retroviruses

Our previous work demonstrated inhibition of transposition by targeting *E. coli* RNase HI to

TY-1, a yeast transposon whose replication is very similar to that of mammalian retroviruses. We have performed two types of experiments using different retroviruses and experimental approaches to address whether *E. coli* RNase HI can be used to inhibit multiplication of mammalian retroviruses. In the first set of experiments, performed in collaboration with Dr. Jef Boeke of Johns Hopkins University, MuLV was used. Boeke's lab had previously shown that a capsid-SN (staphylococcal nuclease) fusion can inhibit viral multiplication. Using a similar approach, we constructed genes which encode capsid-*E. coli* RNase HI fusion proteins, transfected them into chick embryo fibroblasts and infected them with MoMuLV. Inhibition of viral multiplication was very similar to that observed using the capsid-SN fusion protein. Thus, *E. coli* RNase HI may be useful for inhibiton of retroviral multiplication in mammalian systems. We also wish to know at which step (presumably) in replication *E. coli* RNase HI acts. To ascertain the site of action, it would be useful to have greater than 99% inhibition of viral multiplication. We found that for TY-1 inhibition, the amount of RNase HI protein expressed affected the fraction of TY-1 transposition; with low levels, we observed about 80% inhibition whereas at high levels we achieved greater than 99% inhibition. To increase the level of inhibition, we can employ other promoters (RSV was used in the experiments described here). It would be useful to try a cell-type specific promoter in certain experiments, particullarly for HIV.

A different approach has been taken for studies involving *E. coli* RNase HI and HIV. Stably transformed Jurkat cells have been constructed which carry genes in which *E. coli* RNase HI is expressed as a fusion protein with the HIV GAG protein. Very recent data indicate that two of the cell lines derived from a single trasfection experiment generate virus particles that, upon infection of a second host not carrying a GAG-*E. coli* RNase HI gene, produce particles (extracellular p24) but contain little or no HIV RNA. These experiments need substantial work to demostrate that the fusion protein is being expressed and that inhibition is a result of such expression. All of the transformants generated thus far are derived from the wild-type HIV-1 GAG gene. It is known that export of the GAG mRNA from the nucleus to cytoplasm requires a cis-acting RRE sequence and the rev protein. Those cell lines which fail to inhibit normal viral multiplication may do so due to lack of mRNA transport. We have added an RRE element to our plasmid DNAs used for generating stable transfectants and will infect cells in which rev is being constitutively expressed (CEM-TART). The best estimate of when these experiments will be completed (assay of HIV-1 infection) is Nov. 1, 1996. These experiments require that the clones have the correct sequence (in progress now), stable transformants to be selected and clonal cell lines generated (four-six weeks) and infection with HIV-1 and subsequent analysis. Cell line development and viral infection and analysis are being performed under contract at the Frederick Research Center (company). Each plasmid transfection, clonal cell isolation and viral infection-analysis cost about $7,750.

Once the data are collected (demonstrating inhibition - based on our preliminary studies described above, including those with TY-1 and MuLV), we need to fine-tune the system. What if we use a different promoter (T-cell specific, CMV or other)? What if we use an RNase H other than *E. coli* RNase HI (*S. cerevisiae*, *S. pombe* or human)? These experiments are time consuming and relatively expensive but could permit us to obtain high levels of inhibition making analysis of the point in replication where RNase H acts easier to determine.

To carry out experiments in the human cells, we will express *E. coli* RNase H fused to the gag protein of HIV-1 by using plasmid vectors that can replicate in *E. coli* and mammalian cells. Each plasmid will be introduced into CD4+ cells and the effect of expressing the fusion protein on HIV-1 infection will be assessed. If we get results suggesting that *E. coli* RNase H can also interfere with HIV-1 replication, we will attempt to determine the substrate that the RNase H recognizes by looking at various replication intermediates. Such inhibition would be useful both as a tool for understanding replication of HIV-1 and, possibly, could be used for gene therapy of patients with the HIV-1 virus. These experiments require the use of plasmids stably transfected into CD4+ cells and then a variety of tests to see an effect on HIV-1 infection. Facilities and some personnel for these experiments will be obtained by use of a contract with a company such as Frederick Research Center in Frederick, Maryland. The following steps are required for these experiments: (1) Transfection with the appropriate plasmid, selection for drug resistance (neomycin) gene encoded on the plasmid, cell culture for establishing stably expressing cell line and verification of the presence of the expressed protein (2) Single-cell cloning from mixed cell population and expansion of cell lines that are stably expressing the protein (3) characterization of the expression of several of the cell lines to determine the level of expression of the protein in each of the cell lines and (4) Time-course assays following infection of the cells with HIV-1.

Expression of gag-RNase H fusions will be: (1) vector, (2) wild-type RNase H of *S. cerevisiae,* RSV promoter (3) wild-type RNase H of *S. pombe,* RSV promoter (4) same as (2) but CMV promoter (5) *E. coli* RNase H using CMV promoter (6) same as (2) using thymus specific promoter such as the CD2 gene promoter-enhancer, (7) same as (5) using thymus specific promoter (8) RNase H2 of mouse with RSV promoter (9) human RNase H2 with RSV promoter and (10) human RNase H2 using thymus specific promoter.

Completion of the four steps described above will require three to four months after the experiments are initiated. The cost (Frederick Research Center) of establishment of a several isolates of cell lines producing the RNase H from a single plasmid is $5,810. Time-course assays of reverse transcriptase for these will be $2,065 for a total of $7,875 or for all 10 described above about $78,000. The cell lines will already be established at that point so the cost per assay should be on the same order as the reverse transcriptase assay (i.e., about $2,000 each). These experiments will be continued in the second year and the exact nature of the experiments will be based on the results obtained during year one.

II. Cellular RNases H

A) Yeast RNase H1

Interaction between proteins

1. Two-hybrid system

A clever and sensitive method for examining protein-protein interactions is available in *S. cerevisiae*. The "two-hybrid" method relies on activation of transcription of a certain gene in *S. cerevisiae* that requires an activator protein. We are interested in using this method for several reasons. First, it is possible that the RNase H is a homopolymeric dimer. We can test this by cloning the complete gene encoding RNase H1 into each of the two vectors used in the two-hybrid system. One complication can arise from this type of experiment when there is sufficient contribution of DNA binding to cause activation of transcription. If this artifact occurs, we will not be able to use the two hybrid system on the wild-type protein but it may be possible to substitute any of several mutant proteins that are defective in binding double-stranded RNA or RNA-DNA hybrids. If we can perform these experiments, we should be able to define the region of the proteins that interact to form dimers. Second, the RNase H1 protein has two domains that influence the activity of the other. The simple interpretation of these results is that the two domains have a direct interaction with each other. If this is this case, we can define these regions of interaction employing the two-hybrid system. Second, we find that the HIV RNase H only domain can functionally complement the growth defect of *rnhA recB* or *recC* strains yet has no activity in vitro. We suspect that the HIV RNase H might be interacting with some *E. coli* protein that contributes to its stability and/or activity. It is possible with the two-hybrid system to look for such interactions by fusing the HIV RNase H with the DNA-binding domain of the activator and screening an *E. coli* library fused to the "activator" domain. The two-hybrid system can also be employed to examine interactions of *S. cerevisiae* proteins with the RNase H1 to help us understand the site(s) of action of the enzyme.

2. Use of the BIAcore to search for interactions between RNases H and cellular components

Use of the BIAcore for measuring interactions between two large molecules is based on an increase in refractive index that is somewhat proportional to the molecular weight of the substance being bound and requires only an interaction to be observed. If we bind RNase H to the surface of the BIAcore chip, we can use the interaction assay to search for proteins that are found in cells that bind to RNase H. In some ways, this is similar to the yeast two-hybrid system. One difference is in the two-hybrid system, the gene producing the interacting protein is obtained. Proteins found using of the BIAcore would have to be identified. If enough protein is obtained, determination of the sequence could lead to the gene by reverse genetics. At a recent BIAsymposium, Randall Nelson of Arizona State University described some very interesting work in which he is able to take a chip from the BIAcore after some interaction has taken place and put it in a Mass Spectrometer and detect the protein bound to the chip. Moreover, he has been working on a technique to treat, in the Mass Spectrometer, very small amounts of protein with carboxypeptidase to sequentially remove amino acids for the C-terminus generating a series of proteins whose sizes differ by one amino acid. The resolving power of the Mass Spectrometer is sufficient to determine amino acid that has been removed and, therefore, the amino acid sequence of the protein that is interacting. Since most of proteins will be of different sizes, it should be possible to see several different proteins in any one assay. This use for the BIAcore to look at complex mixtures as well as protein-nucleic acid interactions is an unusually useful approach. For example, when RNA-DNA hybrids are bound to the surface, RNase H can interact with the RNA-DNA providing a "natural" substrate with which cellular proteins can bind. Alternatively, crude or partially purified fractions can be passed over the RNA-DNA surface and proteins that bind can be determined. Using standard protein purification techniques, the components necessary for complex formation can be obtained. It should be possible to build up complexes, determining not only the composition but also the order in which components are required to be added. At the same BIAsymposium, Charles McHenry's lab reported the assembly of *E. coli* DNA polymerase III holoenzyme on a BIAcore chip, obtaining information on the order of addition as well as the kinetic parameters for the on and off rates. As described under "Interaction of RNases H with dsRNA and RNA-DNA hybrids", we can cross-link use methylene blue and visible light to cross-link dsRNA (and presumably RNA-DNA) to proteins. We will use RNA-DNA hybrids or dsRNAs that have a biotin residue at their 5'-ends. After cross-linking, we can selectively attach those proteins linked to nucleic acid on strepavidin columns, isolate the protein-nucleic acid complex and determine the site(s) on the protein that are cross-linked to nucleic acid.

B) RNase H and dsRNA-binding motif influence each other

RNase H1 of *S. cerevisiae* has two domains, a C-terminal RNase H domain and an N-terminal region that binds to double-stranded RNA and RNA-DNA hybrids. At low Mg^{2+} concentrations, RNase H activity is weak and dsRNA binding is strong while at high Mg^{2+} concentrations the reverse is true. To determine the portions of the two domains that are responsible for the increases in RNase H activity and decreases in dsRNA binding with increasing Mg^{2+} concentrations, we will construct synthetic RNase H1 genes with multiple restriction sites (first for *S. cerevisiae* and then either chicken or human). Portions or all of the RNase H gene will be mutagenized in vitro and plasmids carrying these treated genes will be introduced into *E. coli*. We know that when the full-length protein is expressed in this way, we can perform colony lifts and detect binding to dsRNA. We would expect to obtain mutants whose proteins have lost their ability to bind dsRNA because (i) the protein is not made or (ii) mutations in sites critical for dsRNA binding have been generated. When we include Mg^{2+} in the binding assay, we would expect to see no binding to dsRNA. If this is done in a strain defective for RNase III, we should eliminate any problems due to degradation of the dsRNA by this *E. coli* enzyme. Among the types of mutations that would give this phenotype are frame shift mutations after the dsRNA-binding region (equivalent to our N-176 protein - the first 176 amino acids corresponding to the non-RNAse H region). These should be easy to eliminate from further consideration by showing the a full-length protein is not generated or these plasmid are unable to complement (in vivo assay for RNase H activity) the temperature-sensitive defect of the *rnhA::cat recB270* (Ts) strain. An interesting class of mutations would be those that have modified the interaction between the two domains such that Mg^{2+} is unable to inhibit binding to dsRNA, as is found by using Northwestern assays. One subset of this class might include mutants in the RNase H domain catalytic amino acids (Mg^{2+} binding site) since one interpretation of our results is that it is binding of Mg^{2+} that assists in the conformational change.

Another approach to studying this interaction will make use of the BIAcore. By linking the dsRNA-binding region to the BIAcore chip surface, we can pass solutions containing all or portions of the RNase H protein over this surface and measure interactions. We can vary the source of the RNase H (*S. cerevisiae*, *S. pombe*, *E. coli* or others) and determine if the interaction is limited to the homologous enzyme. It might be worth noting here that *S. cerevisiae* RNase H1 has an insertion of 11 amino acids just prior to β_5 and could create a region for specialized interactions. An insertion is also found at the corresponding positions in *C. fasciculata C. elegans* and chicken RNase H. However, *S. pombe* RNase H1 has no such insertion yet its binding to dsRNA is sensitive to Mg^{2+} concentrations. For interactions to occur between the domains it may be important to have dsRNA bound to the dsRNA-binding region prior to passage of the soluble proteins over the surface. As described under "Interaction of RNases H with dsRNA and RNA-DNA hybrids", we can cross-link use methylene blue and visible light to cross-link dsRNA (and presumably RNA-DNA) to proteins. We will use RNA-DNA hybrids or dsRNAs that have a biotin residue at their 5'-ends. After cross-linking, we can selectively attach those proteins linked to nucleic acid on streptavidin chips and measure interactions between the protein-nucleic acid complex and other parts of the protein or other proteins.

C) Interaction of RNases H with dsRNA and RNA-DNA hybrids

We can use methylene blue and visible light to cross-link dsRNA (and presumably RNA-DNA) to proteins. We will use RNA-DNA hybrids or dsRNAs that have a biotin residue at their 5'-ends. After cross-linking, we can selectively attach those proteins linked to nucleic acid on strepavidin columns, isolate the protein-nucleic acid complex and determine the site(s) on the protein that are cross-linked to nucleic acid. This technique may also prove useful for NMR studies. We have a 50 amino acid residue protein corresponding to the first 50 residues in *S. cerevisiae* RNase H1 which appears disordered in solution. We will try to cross-link this peptide to RNA-DNA or dsRNA to determine if by doing so, a stable structure is formed. Clearly, we would like to form a unique structure amenable to NMR analysis. Therefore, the size and content of the nucleic acid used for these experiments needs to be varied to determine the best nucleic acid to employ. Based on BIAcore studies, we already know that *E. coli* RNase HI binds (or covers) nine base pairs of RNA-DNA hybrid. For the larger yeast enzymes, we can determine the binding size of the full-length protein, the dsRNA-binding region or the RNase H region. These cross-linking experiments can also be performed using reverse transcriptase of HIV and MuLV.

D) Other RNases H

DNA sequences deposited in the data bases as a result of various genome and cDNA sequencing projects include three RNase HI-like RNase H genes from *C. elegans* and several RNase HII-like genes from human mouse, *S. cerevisiae*, and *C. elegans*. A single RNase HI-like gene has been reported from human cDNA sequences but we have been unable to verify the human RNase H1 gene; neither the clone nor human DNA yields PCR products predicted from the deposited sequence. However, RNase HI-like genes are present in *C. elegans* genomic DNA(three genes) and several RNase HII-like genes have been found in cDNAs from mouse and one from human. We have the cDNA clone reported for human RNase H2, and we have confirmed that it has the reported sequence. We will study the expression of the mRNA and proteins for all of these genes especially for *C. elegans* for which we can determine the cell types expressing these three RNase HI-like enzymes and their cellular localizations. These results will tell us at least how many genes of this type there might be in higher organsims (*S. cerevisiae* seems to have only one) and how a drug that inhibits RNases H (including retroviral RNase H) might affect cell growth and development.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00069-24 LMG

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Genetics of Mammalian Retrovirus Replication

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	J.G. Levin	Section Chief	LMG:NICHD
Others:	J. Guo	IRTA Fellow	LMG:NICHD
	M. Powell	IRTA Fellow;Staff Fellow	LMG:NICHD
	T. Wu	IRTA Fellow	LMG:NICHD
	W. Wu	Visiting Fellow	LMG:NICHD
	Y.-X. Feng	Guest Worker	LMG:NICHD
	K. Post	Biologist	LMG:NICHD

COOPERATING UNITS *(if any)*

ABL Basic Research Program, NCI-FCRDC (A. Rein, T.D. Copeland); SAIC Frederick, NCI-FCRDC (L. Henderson, R. Gorelick); Case Western Reserve Univ. (S. LeGrice)

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Section on Viral Gene Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.7	3.7	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to define the molecular mechanisms involved in the replication of mammalian retroviruses and in particular, to understand the factors which influence the regulated expression of viral genetic information. We have been using an in vitro approach to study events in reverse transcription and the role of the viral nucleocapsid protein (NC) in increasing the efficiency of viral DNA synthesis. We have shown that HIV-1 NC reduces reverse transcriptase (RT) pausing near the murine leukemia virus polypurine tract (PPT) during elongation of (-) strand DNA. Mutational analysis indicates that the features of HIV-1 NC which contribute to NC activity include: the basic residues flanking the N-terminal zinc finger, the zinc finger structures, and the cysteine and aromatic amino acids within both fingers. We have also developed an in vitro HIV-1 system to study the first strand-transfer, which occurs after synthesis of (-) strong stop (SS)DNA. Using donor and acceptor RNAs containing TAR (a large stem-loop in the terminal repeat region which could interfere with annealing of (-) SSDNA and acceptor), we observe that the products are largely heterogeneous (+) strand DNAs formed by a self-priming mechanism from (-) SSDNA; in this case, transfer efficiency is only 2%. Deletion of TAR or addition of NC greatly stimulates strand-transfer and drastically reduces self-priming. Thus, NC overcomes the potential inhibitory effect of TAR, while allowing the virus to maintain TAR for subsequent transactivating activity. Experiments to determine whether self-priming can be detected in endogenous reactions with HIV-1 virions are in progress. In other work, we have analyzed HIV-1 RT mutants having alanine substitutions in the "primer grip" region. These mutants can extend DNA primers, but most of the mutants cannot utilize RNA primers for initiation of (+) and (-) strand DNA synthesis; several mutants are also deficient in specific RNase H cleavage at the 3′ terminus of the PPT. We conclude that the primer grip region plays a major role in recognition of primer-template structures containing the PPT or $tRNA^{Lys3}$ RNA primers. Other studies have focused on a model system which mimics NC-mediated maturation of the genomic RNA dimer in virions. We find that the basic residues flanking the N-terminal zinc finger and the phenylalanine in the first finger are critical for NC function. Surprisingly, the conserved zinc finger structures themselves are not required.

Project Description:

Objectives: To define in molecular terms the mechanisms involved in the replication of mammalian retroviruses, in particular, murine leukemia virus (MuLV) and human immunodeficiency virus (HIV); to study factors which influence the regulation and expression of viral genetic information; to understand the relationship between genetic structure and functional activity in virus infection.

Methods Employed: Recombinant DNA technology is used for molecular cloning of viral genes. DNA fragments to be cloned are amplified by the polymerase chain reaction (PCR) procedure. Cloned viral DNAs are expressed in *E. coli* or in some experiments, are transferred to mammalian cells by standard transfection techniques. Viral and plasmid DNAs are analyzed by restriction endonuclease digestion followed by agarose gel electrophoresis. DNA sequence analysis is carried out by the dideoxy-chain termination method. Viral DNA sequences cloned in expression vectors are transcribed with either SP6 or T7 RNA polymerase. The T7 transcripts are used for analysis of RNase H activity and as templates in primer extension assays. In some cases, SP6 and T7 transcripts from the same DNA plasmid are hybridized to form a substrate for assay of a retroviral dsRNA nuclease activity. Bacterially expressed reverse transcriptase (RT) is purified by chromatography on HPLC and Sephacryl S-200 columns. Viral proteins are analyzed by immunoprecipitation or by immunoblotting techniques. SP6 transcripts are used for studies on in vitro dimerization of viral RNA. Dimer formation is analyzed by elecrophoresis in 2% Metaphor gels followed by ethidium bromide staining; for labeled RNAs, phosphorimager analysis is used.

Major Findings:

1. Studies on the Mechanism of Reverse Transcription.

A. Initiation of (+) strand DNA Synthesis by the Polypurine Tract (PPT) Primer. We have developed a simple in vitro oligo assay to investigate HIV-1 (+) strand priming. Results obtained with this assay taken together with data from CD spectroscopy led us to conclude that the nucleotide sequence at the 3' end of the PPT (a run of 6 G's) and an unusual helical structure are essential for PPT function.

We are now using the oligo assay to investigate the protein interactions involved in specific recognition of the PPT by HIV-1 RT. In current work, we are studying the activity of alanine-scanning mutants of the "primer grip" region in the p66 subunit of HIV-1 RT. These residues are of special interest since they interact with bases at the 3' end of the primer i.e., precisely those found to be critical for PPT function. All of the mutant RTs with one exception can extend a 15-nt DNA version of the PPT as efficiently as WT RT. In contrast, only three of the mutants are able to prime (+) strand synthesis with the 15-nt RNA PPT. The mutants all exhibit RNase H activity, although several have lower levels of activity than WT RT and in one case, cleavage is not precise. Band-shift experiments indicate that the difference in activity with RNA and DNA PPT primers is not correlated with more efficient binding of the RTs to the DNA PPT, since the mutants bind to both primers to approximately the same extent. Interestingly, most of the primer grip mutants are also unable to initiate (-) strand DNA synthesis with an 18-nt RNA oligo (consisting of the bases at the 3' end of the tRNA primer). However, an 18-nt DNA version of the tRNA oligo is readily extended by all of the mutants, except the one which is also defective in extension of the DNA PPT.

The differential activities with RNA and DNA primers may reflect differences in RT recognition of the helical structures of RNA-RNA or RNA-DNA primer-templates and hybrids containing a DNA primer. The results also emphasize the importance of screening potential RT inhibitors in assays which measure their ability to inhibit interactions with the two RNA primers, in addition to commonly used assays with more non-specific primer-templates. (Collaboration with S. Le Grice, Case Western Reserve University)

B. Role of Nucleocapsid (NC) Protein in Reverse Transcription. In earlier work, we showed that RT is not completely processive. Since the efficiency of DNA synthesis has a direct effect on the efficiency of virus replication, we predicted that cellular and/or viral accessory proteins are recruited to increase the rate and extent of reverse transcription during infection. Initial studies demonstrated that NC, a single-stranded nucleic acid binding protein, can fulfill this function.

1. Reduction of RT pausing by HIV-1 NC during synthesis of MuLV (-) strand DNA. During (-) strand elongation on an MuLV RNA template, there is a major RT pause site downstream of the PPT. As reported last year, a stem-loop structure in the RNA template is responsible for the observed pausing. HIV-1 NC destabilizes this structure: Thus, NC dramatically reduces pausing and stimulates synthesis of full-length DNA, resulting in an increase in the efficiency of viral DNA synthesis.

HIV-1 NC has two zinc fingers, each with a highly conserved Cys-His motif; both zinc fingers are required for viral RNA packaging and infectivity. Results from mutational analysis demonstrate that the zinc finger structures and certain residues therein contribute to NC activity in the pausing system. For example, NC can function with only one zinc finger, although not as efficiently as the WT protein. This conclusion is based on experiments with (i) NC mutants having a deletion of either zinc finger or (ii) recombinant NC modified with 3 equivalents of N-ethylmaleimide (NEM), which specifically inactivates the 3 Cys in the C-terminal finger. (NEM is similar to other less toxic drugs currently being evaluated for anti-HIV therapy in animal trials.) If the ability to coordinate zinc is completely eliminated, as in an NC having the 6 Cys replaced with Ala or an NC modified with 6 NEM, there is an even lower level of activity. Interestingly, NC mutants with two copies of the first or second fingers (1:1 or 2:2, respectively) or with the two fingers in reversed order (2:1) exhibit essentially wild-type activity. These results indicate that the two zinc fingers have equivalent activity in the pausing assay. In addition to the 6 Cys, the conserved aromatic residues in each zinc finger are also important for activity. NC activity is absolutely dependent on the presence of the 8 basic residues which flank the N-terminal zinc finger, reflecting the critical role of these amino acids in nucleic acid binding. The observation that coordination of zinc is important for NC function during (-) strand DNA synthesis is significant, as it raises the possibility that HIV reverse transcription could be targeted by a new class of drugs which inactivate or destroy the zinc fingers of NC. (Collaboration with L. Henderson, T. D. Copeland, R. Gorelick, and A. Rein, FCRDC)

2. First Strand-Transfer. Following synthesis of (-) strong-stop (SS) DNA, there is a strand-transfer event which is facilitated by pairing of the R sequences at the 3' end of the DNA and the 3' end of viral RNA. A large secondary structure formed by the first 62 nt of R, present in the RNA (where it is known as TAR) and also in the complementary sequences in (-) SSDNA, has the potential to interfere with the annealing reaction and thereby reduce the efficiency of strand-transfer. This year we developed an in vitro HIV-1 strand-transfer system to investigate how the virus maintains an intact TAR sequence (which is necessary for the transactivating activity of Tat), without compromising the efficiency of DNA synthesis.

Using HIV-1 donor and acceptor RNA templates containing all of R and part of U5 and U3, respectively, we observe synthesis of (-) SSDNA, the transfer product, and large amounts of a class of heterogeneous (+) strand DNAs designated as "SP" products. We have established that the SP products are synthesized by a self-priming mechanism from (-) SSDNA: A TAR-related secondary structure in the DNA induces self-annealing at the 3' end of the DNA, allowing the (-) SSDNA to serve as both primer and template for synthesis of (+) strand products. This conclusion is supported by sequence analysis of SP products and other lines of evidence. Thus, formation of SP products (i) occurs with a lag after synthesis of (-) SSDNA; (ii) occurs in the absence of acceptor; (iii) is inhibited by actinomycin D; and (iv) requires RNase H degradation of donor RNA. In addition, mutations which destroy the TAR structure can drastically reduce formation of SP products.

Transfer efficiency in reactions with RNA templates containing TAR is ~2%; deletion of most of TAR increases the efficiency to ~30%. These observations indicate that TAR-induced synthesis of SP products reduces the efficiency of strand-transfer. Using WT templates, we find that NC stimulates strand-transfer in a dose-dependent manner (up to ~10-fold), while also reducing the amount of SP products (~10-fold). This result is consistent with the ability of NC to destabilize secondary structures.

From this work, we conclude that a major role of NC is to overcome the potential inhibitory effect of the TAR structure on first strand-transfer, while allowing the virus to maintain TAR for transcriptional activation during a later stage in infection. (Collaboration with L. Henderson, FCRDC)

2. The Role of Nucleocapsid (NC) Protein in Maturation of Retroviral RNA Dimers. After a virus particle is released from the cell, the genomic RNA dimer undergoes a conformational change, termed "maturation" of the dimer, which is mediated by NC. Maturation within a retrovirus particle is the first known manifestation of nucleic acid chaperone activity in vivo. We now report further results with a system which represents a simple, well-defined model for maturation in vitro.

In earlier work, we reported that a 342-nt transcript from the 5' end of the Harvey sarcoma virus (HaSV) genome can form two distinct dimers differing in thermostability. Treatment of the less stable dimer with HIV-1 NC (under conditions which reflect the NC:RNA ratio in the virus particle) converts it to the more stable form. This result is consistent with the idea that NC stabilizes the dimers by virtue of its RNA chaperone activity, transiently breaking intramolecular bonds in the less thermostable dimer so that it can be converted to the more stable form. Although NC functions as a nucleic acid chaperone in both the maturation and pausing systems, the results obtained with the NC mutants differ in some respects. In the case of maturation, the basic residues flanking the N-terminal finger as well as the aromatic residue between the first two Cys in the N-terminal finger are absolutely required for activity. The aromatic residue is normally Phe, but it can be replaced by Trp, since the 2:1 and 2:2 mutants (see above) are active in the assay. In contrast to the results on pausing, the zinc finger structures themselves are not required, since replacement of the 6 zinc-coordinating Cys with Ala does not abolish activity. Residues within the C-terminal finger also appear to be unnecessary.

From these findings we conclude that the conserved features of NC which are essential for maturation include: an aromatic residue in the context of a highly basic environment. (Collaboration with A. Rein, FCRDC)

Proposed Course of Project:

Reverse Transcription. The major focus of our work during the coming year will be the elucidation of reactions which are required for synthesis of viral DNA, structure-function analysis of RT, and investigation of the role of the NC protein as an accessory factor in reverse transcription.

1. Initiation of (+) Strand DNA Synthesis.

A. In vitro (+) strand priming. (i) The role of bases at the 5' end of the PPT has not yet been defined. In our oligo assay, we have been using a substrate whose 5' end corresponds to the last 5' base of the PPT. To determine whether proper processing at the 5' end of the PPT is a prerequisite for cleavage at the 3' terminus, we will use our in vitro assays to study primer generation and activity with substrates having additional sequences upstream of the PPT. (ii) Further analysis of HIV-1 RT primer grip mutants is currently focused on the effect of single- and double-mutations which substitute other aromatic residues at positions 229 (normally Trp) and 232 (normally Tyr). These residues are of special interest since the W229A mutant is defective in polymerase activity and RNase H cleavage by Y232A is not precise. Since residues in the primer grip form one side of a binding pocket of the non-nucleoside inhibitor nevirapine, we will also test the activity of this drug on (+) strand priming. Additional information on the mode of action of nevirapine should be useful, as it has just been approved by the FDA for anti-HIV therapy. (iii) Dr. S. Wilson (NIEHS) has sent us a series of mutants with alanine substitutions in residues of the αH and αI helices in the thumb subdomain, which are important for positioning of the primer-template. We will characterize the mutants with respect to their priming activity with RNA and DNA PPT primers, RNase H activity, and ability to bind RNA and DNA primers.

B. Requirements for (+) strand priming in vivo. (i) We plan to study the requirements for (+) strand priming in HIV-1 infected cells to verify the results obtained in the in vitro assays. For this study, we will construct molecular clones of HIV-1 (pNL4-3) having mutations in the PPT sequence. Mutants will be transfected into non-permissive HeLa cells. Virions will be produced after a single-cycle and will be tested for infectivity in a T cell line at various times post-infection using an assay for RT. Cells producing non-infectious virus will be analyzed for the presence of (+) and (-) strand DNA products by PCR. In complementary experiments, we will use saturation mutagenesis to provide a positive in vivo selection of sequences with priming activity. Since there are no facilities for HIV work in Building 6, we will perform all cell culture work in Building 4. (Collaboration with E. Freed, NIAID). (ii) The requirements for in vivo (+) strand priming by HIV and a murine virus will be compared. PPT mutations will be generated in the Harvey sarcoma virus (HaSV) genome: its PPT is smilar to that of MuLV and is recognized by MuLV RT; in addition, HaSV carries the *ras* gene, which induces focus formation, a convenient marker for replication-competent virus. NIH3T3 cells will be infected with supernatant fluids from an MuLV packaging cell line transiently transfected with HaSV clones. Foci will be scored and PCR analysis will be used to monitor (+) strand DNA synthesis at various times post-infection. (Collaboration with A. Rein, FCRDC)

2. First Strand-Transfer. Results obtained thus far with a model system demonstrate that NC increases the efficiency of the first strand-transfer, at least in part, by destabilizing the TAR-related secondary structure in (-) SSDNA. In future work, in vitro and in vivo experiments are planned to address the following issues. (i) In an effort to identify elements within NC which are functionally significant for the first strand-transfer, we will test the activity of a unique series of NC mutants in our in vitro assay. Initial results indicate that an NC in which the basic residues flanking the N-terminal zinc finger have been changed to Ala is completely inactive. Other mutants to be tested include: synthetic NC's with a deletion of either the first or second zinc finger or Ala substitutions in the Cys or aromatic residues in each zinc finger; and recombinant NC's with a duplication of each motif (1:1 and 2:2) or reversal of the order of the motifs (2:1). The activity of HIV-1 and other NC's will be compared. The effect of chemical modification of HIV-1 NC with NEM will also be tested. (ii) We will determine whether the (+) strand SP products, which are generated in vitro by self-priming from (-) SSDNA, can be detected in endogenous reactions using detergent-treated HIV-1 virions. Our approach will be to analyze the time course for synthesis of (-) SSDNA, (+) SSDNA, and longer (-) and (+) strand DNAs. Polyacrylamide and agarose gels will be used to achieve high resolution of short and long products, respectively. Based on the in vitro results, we would predict that SP products will not be detected in reactions with wild-type virions, since they contain NC. (Collaboration with J. Bess, FCRDC). (iii) We have recently found that actinomycin D (Act D), a drug which intercalates between G's in DNA, abolishes strand-transfer in our model system, even in the presence of NC. Temin showed that Act D

inhibits de novo retroviral infection and therefore limits virus spread. Thus, this drug, already approved by FDA for treatment of certain tumors, or less toxic analogues (some of which are already available) may have potential use in anti-HIV therapy. To determine whether the first strand-transfer is the target of Act D during reverse transcription, we will test the effect of Act D on synthesis of DNA products in an endogenous reaction. We will look for accumulation of (-) SSDNA as well as inhibition of synthesis of (+) SSDNA and (-) strand DNAs which are longer than (-) SSDNA. We also plan to do cell culture experiments. DNA products in the Hirt supernatant fraction of cells treated with Act D at the time of infection or at various times post-infection will be analyzed by PCR. In an in vitro approach, we will test the effect of Act D on the annealing reaction between (-) SSDNA and acceptor RNA.

3. Second Strand-Transfer. During reverse transcription, there is a second template switch which is necessary for synthesis of the left LTR and full-length double-stranded linear DNA. We are now developing an HIV-1 model system which reproduces the events associated with the second strand-transfer. The donor is a “chimeric” (-) strand DNA-RNA oligonucleotide containing the DNA sequence from the 3' end of U5 and the RNA sequence (18 nt) is from the 3' terminus of the $tRNA^{Lys3}$ primer. Using a DNA primer, the donor will serve as a template for synthesis of a truncated version of (+) strand strong-stop DNA. The acceptor, a shortened version of (-) strand DNA, contains the 18-nt complement of the primer binding site (PBS) sequence and downstream bases. Several reactions can take place with these templates: (i) synthesis of (+) strand DNA; (ii) removal of the tRNA sequence by RNase H; (iii) transfer of (+) strand DNA to the acceptor template and strand displacement synthesis; and (iv) elongation of (+) and (-) strand DNAs to give a linear double-stranded product. Using appropriate conditions, each of these reactions can be studied separately. We plan to est the effect of Act D on these reactions. A possible role for NC in second strand-transfer will also be investigated. Since NC would be expected to promote the annealing of the (+) and (-) strand DNAs, we anticipate that it would increase the efficiency of strand transfer. If this prediction proves to be correct, we will test the activity of the NC mutants described above. We will also test the activity of heterologous NC proteins.

Significance to Biomedical Research and the Program of the Institute:

The advantages of viral systems as models for studying the regulation and expression of genetic information are well recognized. Studies on retroviruses are of special interest since these viruses establish a chronic infection in the host cell by integrating viral genetic information into the host chromosome, in some instances disrupting normal gene function. An understanding of the various steps involved in virus replication is crucial for the success of efforts to limit the pathogenic potential of these agents. More specific considerations follow. (i) Among molecular biologists there is wide interest in the process of reverse transcription. Evidence from non-viral systems indicates that processed genes, certain highly repeated short mammalian DNA segments, and genes for small nuclear RNAs may have been generated by such a mechanism. It has also been known for some time that many eukaryotic transposable elements have a structural organization remarkably similar to that of the retroviruses. The discovery that yeast Ty elements encode a functional reverse transcriptase and that an RNA intermediate is involved in transposition of the element, has lent strong support to the idea that transfer of information from RNA to DNA has been an important factor in the development of the eukaryotic genome. It is interesting that reverse transcriptase is also found in certain bacteria. (ii) The clinical importance of retroviruses should also be emphasized. Retroviral vectors have been considered for some time for use in somatic gene therapy and a number of clinical trials with such vectors are now underway. In addition, public attention continues to be urgently focused on the AIDS epidemic. Studies on the MuLV system have been invaluable for elucidating the molecular mechanisms involved in the replication of the human retrovirus, HIV, the etiological agent responsible for AIDS. At present, reverse transcriptase is a major target of anti-HIV drug therapy for infected individuals. Drugs which interfere with the zinc-coordinating activity of nucleocapsid protein are currently being tested in animal trials and have the potential to provide a new and powerful approach to limit HIV infection.

Publications:

Feng YX, Copeland TD, Henderson LE, Gorelick RJ, Bosche WJ, Levin JG, Rein A. HIV-1 nucleocapsid protein induces “maturation” of dimeric retroviral RNA in vitro, Proc Natl Acad Sci USA 1996;93:7577-81.

Powell MD, Levin JG. Sequence and structural determinants required for priming of plus-strand DNA synthesis by the human immunodeficiency virus type 1 polypurine tract, J Virol 1996;70:5288-96.

Wu W, Henderson LE, Copeland TD, Gorelick RJ, Bosche WJ, Rein A, Levin JG. Human immunodeficiency virus type 1 nucleocapsid protein reduces reverse transcriptase pausing at a secondary structure near the murine leukemia virus polypurine tract, J Virol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01002-14 LMG

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene Expression During Embryonic Development of Xenopus Laevis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	I.B. Dawid	Head (All Personnel Listed LMG:NICHD)		
Others:	A. Karavanov	Vis. Sci.	M. Rebagliati	IRTA Fellow
	M. Taira	Vis. Sci.	R. Toyama	Vis. Associate
	M. Rebbert	Chemist	M. Kobayashi	Guest Researcher
	J-P.Saint-Jeannet	Vis. Fellow	P. Curtiss	Biologist
	E. Glasgow	NRC Fellow	J. Levine	Summer IRTA
	J. Breen	NRC Fellow	R. Wu	Guest Researcher
	B. Huang	Summer IRTA		

COOPERATING UNITS *(if any)*

H. Westphal, LMGD, NICHD; K. Ozato, LMGR, NICHD; K. Cho, UC Irvine; Xi He & H. Varmus, NIH

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Section on Developmental Biology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
11.1	8.5	2.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This laboratory has studied molecular basis of embryogenesis in the *Xenopus laevis* and the zebrafish, with special emphasis on early pattern formation and axis determination. These events are thought to be controlled by cell-to-cell signaling and by the spatially and temporally regulated action of transcription factors.

The Wnt family of signaling molecules is involved in early pattern formation in several ways. We have shown previously that the Wnt signaling cascade regulates the initial dorsoventral polarization in the embryo. During the past year we have studied relationships between different Wnt factors and certain Frizzled proteins, recently identified as Wnt receptors. Further, we analyzed Wnt factor function in neural patterning. Various studies have shown that the initial neuralizing signal leads to the formation of anterior structures; the mechanism of conversion to more posterior cell types is thus an important question. Other workers have suggested that certain Wnt factors have a role in posteriorization. Our recent work has made two main points. (i) In contrast to what was previously believed, there is no equivalence of function between the Wnt family members Wnt3a and Wnt8; while the former effectively converts anterior neural tissue to more posterior tissue, the latter does not. (ii) Beyond its ability to effect posteriorization Wnt3a specifically induces the neural crest.

The second focus of our studies is the function of the LIM class homeodomain protein Xlim-1 in the Spemann organizer region of the gastrula embryo. We have implicated Xlim-1 in neural and muscle induction, and have shown that an activated form of Xlim-1 can stimulate the transcription of the homeobox gene *goosecoid* (*gsc*). More recently we have studied the mechanism of regulation of *gsc* by Xlim-1. We suggest that the *gsc* gene is maintained in an inactive state by a repressor. While mutated Xlim-1 protein is an activator of *gsc*, the wild type protein cannot act by itself but is effective in combination with Otx2. The homeodomain protein Otx2 appears capable of displacing the repressor, allowing the wild type Xlim-1 to activate the *gsc* gene. These studies begin to illuminate the regulatory interactions that lead to the patterning of the gastrula embryo.

Project Description:

Objectives:

This project aims to provide insights into molecular mechanisms during early animal development. *Xenopus laevis* has been chosen as the main experimental system because it is a vertebrate with easily accessible and well-studied embryos. Much information is already available on developmental genes in this organism, its eggs and embryos are suitable for a wide variety of experimental manipulations, and many molecular markers have been isolated that are specific for different developmental stages and different regions of the embryo. Thus, considerable background information exists in this system. However, standard genetics cannot be studied effectively in *Xenopus*, and therefore a research program has been initiated in the zebrafish where such studies are possible. A major aspect of these studies concerns the establishment of the body plan and initiation of tissue differentiation, which in vertebrate embryos depends to a very large extent on inductive cell interactions. A major consequence of cell interactions is the activation of regulatory genes in a spatially and temporally specific manner; such events have been studied in both *Xenopus* and the zebrafish. The focus has been on signaling molecules in the Wnt and in the nodal families of genes, and on the LIM class of homeobox genes which encode transcription factors known to be involved in developmental regulation.

Methods Employed:

Many recombinant DNA methods are being applied, including cDNA and genomic DNA cloning, hybridization to RNA and DNA on filters and in solution, restriction site mapping and DNA sequencing, primer extension analysis, polymerase chain reaction (PCR), and S1 nuclease/RNase protection assays. Bacterial expression of the protein products of some of the studied genes has been used to investigate some properties of the proteins and to generate antibody probes. In vitro transcription and translation is used for the characterization of proteins encoded by isolated genes. Cytological methods include in situ hybridization to tissue sections and in whole mounts, and immunochemical staining of tissue sections and whole mounts. Classical embryological manipulation techniques, such as microdissection, cell disaggregation and explant culture are being utilized. Injection of cloned genes and constructs derived from them into embryos is used to study transcriptional control of the genes. Injection of synthetic RNA into oocytes or embryos is used to express the corresponding proteins in vivo for an analysis of their properties and their functions.

Major Findings:

The role of Xlim-1 in neural induction:

We have shown previously that the Xlim-1 protein must be activated by mutation to exert phenotypic effects on the embryo. The activated form, called 3m, is capable of eliciting the formation of a neural inducing molecule when expressed in animal (pre-ectoderm) cell of the embryo. Recently we have shown that a likely mediator of the Xlim-1 effect is the neural inducing factor chordin, discovered by De Robertis and colleagues. Further we showed that neural tissue generated by expression of Xlim-1/3m has anterior character, as seen by the activation of anterior markers like *otx2*. The anterior character of this tissue can be modified by an inducer elicited by the transcription factor Xbra. We have obtained evidence that the Xbra-induced factor is likely to be a member of the FGF family, a group of signaling molecules that are implicated in the control of many developmental processes.

The regulation of *goosecoid* by Xlim-1

We have shown previously that the homeobox gene *goosecoid (gsc)* is activated by the 3m form of Xlim-1. Studies on transcriptional regulation of promoter constructs together with band-shift and DNase protection assays has led to the following model. Xlim-1 can bind to a region in the *gsc* promoter called UE, and this binding is required but not sufficient for activation. A second control region, named DE, is bound in the cell to a hypothetical repressor molecule which prevents the activation of the promoter by Xlim-1. Xlim-1 also has affinity for DE, but this affinity is too low to displace the repressor; however, the activated form 3m is capable of displacing the repressor and causing transcription of the promoter. The repressor can also be displaced by the homeodomain protein Otx-2; while this alone does not lead to gene activation, it allows wild type Xlim-1 to activate the gene. Thus, Xlim-1 and Otx-2 cooperate in activating transcription on the *gsc* promoter.

A novel LIM domain binding protein

In collaboration with Alan Agulnick and Heiner Westphal we are characterizing a family of proteins that interact effectively with the LIM domains of Xlim-1 and other LIM-homeodomain proteins; they were named Ldb. Mouse Ldb-1 was discovered by Agulnick using an overlay method. Our lab has re-isolated the *Xenopus* homolog of Ldb-1 by using the yeast two-hybrid system and the Xlim-1 LIM domains as bait. *Xenopus* Ldb-1 is 96% identical sequence to mouse Ldb-1; there are no similar proteins in the data base. Thus, Ldb-1 is a highly conserved protein. Ldb-1 functionally complements Xlim-1 in vivo: coinjection of wild type Xlim-1 RNA and Ldb-1 RNA into the ventral side of the embryo generates partial axis duplications; such a phenotype is also seen with the "activated" mutant form of Xlim-1, 3m, but not with wild type Xlim-1 or with Ldb-1 alone.

Cell-cell signalling in the zebrafish gastrula: The role of nodal-related genes

Multiple signaling molecules are required to fine-tune the interactions of different cells in the embryo that lead to the complex pattern formation that takes place during gastrulation. Nodal, a member of the TFG-β family, was discovered in the mouse as a gene required for primitive streak formation. We have isolated three nodal-related (*ndr*) genes from zebrafish and studied two of these in some detail. Both genes, named *ndr4* and *ndr5*, are expressed in early development but in non-overlapping patterns. While *ndr4* is expressed very early and decreases rapidly, *ndr5* arises later, peaks during gastrulation, and continues to be expressed in later embryogenesis. *Ndr4* is localized in the gastrula in a special small population of cells, named the "dorsal forerunner" cells whose function is still being explored, while *ndr5* is expressed in the shield (organizer equivalent) and then in the dorsal axis, implying a role in axis formation. Each of the two genes, when overexpressed in the embryo, leads to ectopic formation of dorsal centers both in the zebrafish and in *Xenopus*. These results implicate the *ndr* genes in the regulation of gastrulation in the zebrafish.

The role of Wnt factors in neural patterning

Wnt factors are secreted signaling molecules that have important roles in many developmental processes. We have shown previously that the Wnt signal transduction cascade in involved in the earliest dorsalizing signal in the *Xenopus* embryo. More recently we have studied the role of Wnt factors in neural patterning.

On the basis of their action in dorsal axis formation and other properties, Wnt factors have been divided into classes which were thought to be essentially equivalent in function. One class, including Wnt8 and Wnt3a, can dorsalize the frog embryo; the other, including Wnt5A and Wnt4, cannot. It was further reported by Moon and colleagues, that Wnt3a can transform anterior neural tissue into more posterior tissue.

We have extended these studies and found that, in contrast to what may have been expected, Wnt8 does not activate the posterior marker Krox20 in neural tissue, while Wnt3a does. The failure of Wnt5a to induce Krox 20 is entirely expected. These results lead to the conclusion that the postulated equivalence classes of Wnt factors are not, in fact, equivalent. Next we asked to what extent Krox20 is a sufficiently specific marker for posterior neural tissue since it is known that this gene is also expressed in the neural crest. We therefore tested two neural crest markers, *slug* and Ap-2; both were induced in anterior neural tissue by the expression of Wnt3a, but not Wnt8. Thus we conclude that Wnt3a, beyond its role in modifying the anteroposterior pattern of the neural plate, is involved in the specification of the neural crest. Similar results were obtained with Wnt1. This role is consistent with the expression of Wnt3a and Wnt1 at the dorsal midline, in the region where neural crest arises.

Wnt Receptors

Recently, the identification of a receptor for wingless (Drosophila Wnt1 homolog) was announced. It is a member of the Frizled family of proteins, of which at least eight members are known in vertebrates. In collaboration with Xi He and Harold Varmus we have used the axis induction assay in frog embryos to attempt matching pairs of Wnt factors and receptors. Wnt5A does not induce a dorsal axis, but the combined expression of Wnt5A and Frizzled 5 (Fz5) leads to the formation of ectopic axes, many of which are complete. We conclude that in vertebrates, as in Drosophila, Frizzled proteins are Wnt receptors, and further that Fz5 is a receptor for Wnt5A.

Significance to Biomedical Research and the Program of the Institute:

Molecular events that occur before and during gastrulation are of critical importance to the entire developmental program of any vertebrate organisms. This project takes advantage of distinct features of the amphibian and fish embryos to study these events in some detail. In particular, it has been clear for a century that inductive interactions are key events for the establishment of body pattern and tissue differentiation in the embryos of all animals and especially in those of vertebrates. Mesoderm induction is the earliest known cell interaction in the vertebrate embryo, followed closely by the elaboration of the nervous system. These events determine the formation of the body axis and the differentiation of major tissues. Therefore a thorough analysis of the molecular mechanisms of axis formation and early neurogenesis is an important part of the overall aim to understand the basis of normal and abnormal embryogenesis. The analysis of regulatory genes that are active during this critical stage of development is a particularly promising approach to this overall aim.

Proposed Course:

These studies will be expanded in several ways. The role of the *Xlim-1* gene in activation of *gsc* is being pursued by further analysis of the specific binding sites of the promoter. Further, the functional interactions of Xlim-1 and Ldb-1 are being studied by coexpression of these proteins in animal explants and testing the response of *gsc* promoter constructs.

The nature and function of the Ldb family of proteins will be studied in several ways. Isolation and characterization of Ldb genes from the zebrafish and *Xenopus* is underway, suggesting that there are at least three members in the family. Their expression patterns and functional role in complementing Xlim-1 will be tested.

The regulation of the *Xlim-1* gene by activin is being studied. A 210 bp fragment has been identified that confers activin responsiveness to the gene. Factors in *Xenopus* embryo extracts that can bind to this regulatory sequence will be looked for by band shift assays, and their binding specificity will be characterized.

The function of Wnt genes is being studied in collaboration with Xi He and H. Varmus. We continue to test for the function of Wnt receptors in the frog embryo, and we plan to isolate the *Xenopus* receptors and characterize their expression patterns in the embryo. Further, the role of Wnt3a in neural crest induction will be studied further.

Publications:

Appel V, Korzh V, Glasgow E, Thor S, Edlund T, Dawid IB, Eisen JS. Motoneuron fate and patterned LIM homeobox gene expression in embryonic zebrafish. Development 1995;121:4117-25.

Karavanov AA, Saint-Jeannet J-P, Karavanova I, Taira M, Dawid IB. The LIM homeodomain protein Lim-1 is widely expressed in neural, neural crest and mesoderm derivatives in vertebrate development. Int J Dev Biol, 1996;40:453-61.

Minucci S, Saint-Jeannet J-P, Toyama R, Scita G, DeLuca LM, Taira M, Levin AA, Ozato K, Dawid IB. Retinoid X receptor-selective ligands produce malformations in *Xenopus* embryos. Proc Nat Acad Sci, 1996;93:1803-7.

O'Connell ML, Rebbert ML, Dawid IB. Isolation of the *Xenopus nrp-1* gene and analysis of its expression following the midblastula transition in injected *Xenopus* embryos. Develop. Growth Differ., in press.

Taira M, Evrard J-L, Steinmetz A, Dawid IB. Classification of LIM proteins. Trends Genet 1995;11:431-2.

Toyama R, Curtiss P, Otani H, Kimura M, Dawid IB, Taira M. The LIM class homeobox gene *lim5*: implied role in CNS patterning in *Xenopus* and Zebrafish. Dev Biol 1995;170:583-93.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01005-09 LMG

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Cellular Proliferation and Diversity in Drosophila

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Kennison, Ph.D.	Senior Staff Fellow	LMG:NICHD
Others:	S. Stoler, Ph.D.	NRC Biotech. Fellow	LMG:NICHD
	M. Adam, Ph.D.	Visiting Fellow	LMG:NICHD
	B. Brizuela	Bio. Lab. Tech	LMG:NICHD
	J. Southworth	Bio. Lab. Tech II, EBON	LMG:NICHD
	R. Scott	Summer IRTA	LMG:NICHD
	R. Charles	Lab Tech, EBON	LMG:NICHD

COOPERATING UNITS *(if any)*

Dr. J. Mueller, Laboratory of Molecular Biology, MRC, Cambridge, England
Dr. M. Vázquez, Laboratory of Molecular Biology, IBT/UNAM, Cuernavaca, Mexico
Dr. J. Tamkun, Department of Biology, University of California, Santa Cruz

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Section on Developmental Biology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.8	2.7	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Genes involved in the determination and differentiation of adult tissues in Drosophila melanogaster have been identified by genetic interactions with homeotic and segmentation genes already known to be required in these tissues. Many new genes required for transcriptional activation or repression of developmental genes have been identified. In addition, we have begun to characterize the cis-acting sequences required for repression of one of the homeotic genes, the Sex combs reduced gene of the Antennapedia complex in order to understand the molecular mechanisms underlying homeotic gene repression and activation.

Certain classes of chromosome rearrangements in the Antennapedia complex cause activation of the repressed wild-type gene on the non-rearranged chromosome. We have used these observations to formulate a model for homeotic gene repression requiring the interaction of multiple silencing elements within the homeotic genes, and have begun characterizing an artificial silencing element based on these predictions. We have also identified a new gene required for the functioning of these silencing elements, which may encode a hunchback-interacting protein involved in the switch from the initiation phase to the maintenance phase of silencing.

One of the genes that we identified as a transcriptional activator of the homeotic genes, the brahma gene, encodes a large nuclear protein conserved from yeast to man. brahma mutations have in turn been used to identify other interacting genes required for transcriptional activation. We have characterized three of these brahma-interacting genes in more detail. All three show strong genetic interactions with brahma mutations and with each other. Two have been transposon-tagged for molecular analysis. We have also shown that the moira gene encodes a transcriptional activator of homeotic genes, but does not appear to be part of the brahma group of interacting genes.

Project Description

Objectives:

During the development of a multicellular organism, cells within the organism acquire specialized functions in reproducible spatial and temporal patterns. These patterns result from both the synthesis of tissue-specific proteins and the differential activation or interaction of pre-existing gene products. All aspects of differentiation must be integrated to form these patterns. The regulation and integration of information depends on intercellular communication within the early embryo, and upon intracellular signalling subsequent to cell-cell interactions.

Our goal continues to be the understanding of the molecular mechanisms that operate to control development. A key step toward this goal is to identify all of the relevant genes, something that is currently only feasible through a combined molecular and genetic approach. The determination of body segments in *Drosophila melanogaster* provides an excellent system for the investigation of the genetic basis of pattern formation. The discovery of homeotic and segmentation regulatory genes, major classes of genes that affect segmental differentiation in Drosophila and are widely conserved among all eukaryotes, has in the past been largely accidental, but the genetic screens that we have performed have been designed to make the search for regulatory genes more systematic. Because of a desire both to identify additional members of the gene networks, and to focus on important regulatory interactions between these genes, screens were designed to identify new loci that are likely to be involved in regulatory interactions with other genes required for segment determination. We have identified a large number of new genes required for proper segment formation, and are characterizing these genes to determine their individual functions in development.

We are characterizing the regulatory steps defective in mutants of the genes identified in our genetic screens. The majority appear to encode transcriptional regulators of multiple homeotic and segmentation genes. One of the new genes required for transcription of the homeotic genes, the *brahma* gene, encodes a large nuclear protein that is conserved from yeast to man. The *brahma* proteins are subunits of a large protein complex that appears to modify chromatin structure to facilitate protein-DNA interactions. We have used genetic interactions with *brahma* mutations to identify other genes involved in this step of transcriptional activation. Because repression (or silencing) of the homeotic genes is as important as their activation, we have also begun to characterize the *cis*-acting elements and the *trans*-acting factors required for silencing of the homeotic genes.

Methods Employed:

Standard methods are used for the culture of Drosophila. The alkylating agent, ethyl methanesulfonate, gamma-ray exposure, and P-element-transposon mobilization are used in the induction and recovery of mutations. The mutations are mapped by meiotic recombination using standard genetic marker mutations or by *in situ* hybridization of labelled nucleic-acid probes to giant polytene chromosomes of the larval salivary gland. Breakpoints of chromosome aberrations are determined by cytological analysis of polytene salivary gland chromosomes or by Southern blot analysis of genomic DNA. Recombinant DNA techniques are employed to isolate and analyze DNA segments from the Drosophila genome. Mutations are mapped within such isolated DNA segments by Southern blotting of DNA from mutant stocks or by DNA sequence analysis. Transcription mapping of chromosome regions is done by Northern blotting and by the analysis of cloned cDNA. Sequence analysis is by enzymatic methods. P-element-mediated mutagenesis and transformation follow the methods of Engels and Spradling and Rubin. Cloned cDNA segments are expressed into protein by the use of fusion vectors with β-galactosidase and antisera are raised by standard methods. Embryos are fixed and stained with antibodies to various Drosophila proteins by the methods of Karr and Carroll or hybridized to labelled nucleic acid probes by the method of Tautz. Larval imaginal discs are fixed and stained with antibodies to Drosophila proteins by the methods of Pattatucci and Kaufman. Analysis of germline function follows the methods of Chou, Noll, and Perrimon.

Major Findings:

As part of our effort to characterize the function of the genes that we have identified as important in regulating expression of homeotic genes, we have continued to characterize mutations that interact with *brahma* mutations. The *brahma* gene encodes a nuclear protein that is conserved from yeast to man. This protein is found in large protein complexes that include at least nine other polypeptides. The yeast homologue, SNF2/SWI2, is a DNA-dependent ATPase that is required for transcriptional activation of multiple target genes. It has been shown to function *in vitro* to enhance binding of specific transcriptional activators to their target DNA sequences when nucleosomes are present. There are at least two different *brahma* homologues in mammals, and these are also found in large protein complexes. Because of the evidence that *brahma* homologues in yeast and man function in large protein complexes, we screened for mutations that interact with *brahma* mutations. We isolated mutations in six other genes, including the *osa* gene that we had already shown was required for homeotic gene transcription. We isolated the *osa* gene and are sequencing cDNA clones to determine the sequence of the putative proteins products. We have mapped another of the interacting mutations, *E(brm)3*, to polytene chromosome bands 67F1-2 and obtained a transposon insertion mutation in this gene. The *E(brm)3* mutation is a late pupal lethal and has a homeotic phenotype that mimics the phenotype of mutations in the *Sex combs reduced* gene of the Antennapedia complex. This is not surprising since the mutation was isolated because of its interactions with *brahma* mutations, which have this same homeotic phenotype. The transposon insertion in *E(brm)3* will allow us to isolate the genomic DNA and begin the molecular analysis of the gene.

We have also begun experiments to examine the molecular basis of homeotic gene silencing. A group of *trans*-acting factors, the Polycomb group, are know to be required for maintenance of homeotic gene silencing. The initiation of silencing requires the segmentation genes, including the *hunchback* segmentation gene. In experiments designed to identify proteins involved in the switch from initiation to maintenance of silencing, J. Mueller and M. Bienz in England identified a protein that interacts with the *hunchback* protein in the yeast two-hybrid system. The Drosophila gene encoding this protein maps to polytene chromosome region 76B-D. We have isolated mutations in twenty essential genes within this chromosomal region and are collaborating with Drs. Mueller and Bienz to identify which of our essential genes encodes their *hunchback*-interacting protein. Our best candidate, *l(3)76BDo*, is a new member of the Polycomb group that we have identified in this region. We have isolated four mutant alleles and all lead to homeotic gene derepression in imaginal cells (a failure of maintenance of silencing). We are now examining homeotic gene expression in embryonic cells to determine at what stage silencing first fails (during the initiation phase, or in the switch from initiation to maintenance).

Current models of homeotic gene silencing are based on the idea of spreading inactivation from a single silencing element within a homeotic gene. Our data from chromosome aberrations within the Antennapedia complex are not consistent with these models for a single site. We have only been able to explain the data with a multiple site model involving DNA looping between silencing elements. Our model makes very specific predictions about a particular mutant chromosome, the *Scx* mutant chromosome. This mutant chromosome has an insertion of repetitive DNA near the *Antennapedia* P1 promoter and derepresses the *Sex combs reduced* promoter that lies 150 kb downstream. Our model makes three very specific predictions that cannot be explained by the other models. The first prediction is that the *Scx* insertion derepresses the *Sex combs reduced* promoter by silencing the *Antennapedia* P2 promoter (which lies between the *Scx* insertion and the *Sex combs reduced* promoter). We have tested this prediction and shown that it is true; the *Antennapedia* P2 promoter is silenced on the *Scx* mutant chromosome as expected. The second prediction of our model is that chromosome aberrations with breakpoints between the *Scx* insertion and the *Antennapedia* P2 promoter should activate the silenced P2 promoter. Our preliminary data suggest that this prediction is true as well. The third prediction is that the repetitive DNA inserted in the *Scx* mutant chromosome binds Polycomb group proteins and behaves as a new silencing element. We have cloned the repetitive DNA from the *Scx* mutant chromosome and have begun sequence analysis. Our preliminary analysis of this sequence suggests that there are small sequence motifs within the *Scx* insertion that are also found within the region of the *Sex combs reduced* gene that we believe contains one of the normal silencing elements.

Significance to Biomedical Research and the Program of the Institute:

Understanding the genetic control of development and its molecular basis is a major aim of the Institute research program. Our work is aimed at understanding the network of genes involved in cellular determination. One aspect of the work, the characterization of the effects on development of mutations in genes involved in transcriptional regulation, should prove invaluable in understanding how cells acquire different fates during embryonic growth. The wealth of genetic material available in Drosophila from the past eighty years of research coupled with recent advances in the techniques for molecular analyses have given Drosophila many advantages among organisms for such a study. The finding of many similarities between Drosophila and mammalian genes has strengthened the belief that studies in Drosophila will lead to a greater understanding of basic problems in development in other animals.

Proposed Course of Project:

We propose to continue our developmental and molecular characterizations of the genes that are required for homeotic gene activation or silencing. Our efforts towards understanding homeotic gene silencing will be directed towards characterizing the novel silencing element associated with the *Scx* mutant chromosome and identifying mutants in the *hunchback*-interacting protein identified by J.

Mueller and M. Bienz. We will continue our molecular characterization of the repetitive DNA insertion in the *Antennapedia* P1 promoter of the *Scx* mutant chromosome. The complete sequence of the insertion will be determined and compared to the 300 kb of Antennapedia complex DNA currently in the database in order to identify other putative silencing elements for the *Sex combs reduced* gene. We will then test these putative silencing elements for their ability to silence a reporter gene in a transgene. We have already confirmed the first prediction from our model for homeotic gene silencing, the silencing of the *Antennapedia* P2 promoter by the *Scx* insertion. We are now testing the second prediction from the model, the activation of the silenced *Antennapedia* P2 promoter by chromosome aberrations with breakpoints between the silenced promoter and the *Scx* insertion. These predictions are unique to our model for homeotic gene silencing by chromosomal looping rather than by the spreading inactivation models currently favored in the field. To further characterize the Polycomb group of *trans*-acting factors required for homeotic gene silencing, we will determine whether the *l(3)76BDo* Polycomb group gene encodes the *hunchback*-interacting protein identified by M. Mueller and M. Bienz. If this gene does not encode the Mueller-Bienz protein, we will continue our characterization of the other essential complementation groups in this chromosomal region to identify the mutants for the *hunchback*-interacting protein.

We will also continue our characterization of some of the genes required for homeotic gene activation. We will continue our molecular analysis of the *osa* and *E(brm)3* genes, and return to a molecular analysis of the *moira* gene. We will use a new method from the Engels lab to generate small deletions flanking a P-element insertion using transposase-induced male recombination. These small deletions can then be used to identify the *moira* transcription unit within our cloned genomic DNA.

Publications:

Kennison JA. The Polycomb and trithorax group genes of Drosophila: Trans-regulators of homeotic gene function, Annual Reviews of Genetics 1995;29:289-303.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01006-08 LMG

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Protein-Nucleic Acid Interactions in Vertebrate Embryogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	T. Sargent	Research Microbiologist (All listed LMG:NICHD)
Others:	M. Morasso	Sr. Staff Fellow
	J. Franklin	IRTA Fellow
	J. Scales	NRC Fellow
	S. Campbell	Biologist
	C. Nocente	IRTA Fellow
	J. Feledy	Clinical Associate

COOPERATING UNITS *(if any)*

K. Mahon, LMGD, NICHD

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Section on Vertebrate Development

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.5	5.75	0.75

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The general aim of this project is to identify and study the function of factors that regulate vertebrate embryonic development. This is being approached in three ways. One approach is to analyze the regulation and function of Dlx3, one of the vertebrate homologs of the Drosophila gene Distal-less. In flies this gene regulates limb and sensory appendage formation. The frog and mouse homologs are both expressed in epidermis, branchial arches, and in gradients in the limbs. The mouse gene is also active in hair follicles, tooth primordia and other locations. DNA elements and protein-DNA interactions responsible for this expression pattern are being mapped. In addition, targeted inactivation and mis-expression studies are being used to test for the function of Dlx3 gene expression in the epidermis of transgenic mouse embryos.

A second approach employed has been to identify mRNAs encoding Eph-related receptor tyrosine kinases that are expressed in specific tissues of early frog embryos. One such kinase, called Pagliaccio, has been shown to affect cell-cell adhesion when constitutively activated in Xenopus embryonic ectoderm, probably by modulating cadherin function. A related gene, TCK, is also being studied.

The third approach focuses on the developmental function of calcium-dependent cell adhesion molecules in the zebrafish neural tube. A novel cadherin, VN-cad, has been cloned, sequenced, and characterized by in situ hybridization. Functional studies are being carried out by injection of VN-cad messenger RNA into zebrafish embryos.

Project Description

Objectives:

The overall objective of this project is to elucidate molecular mechanisms that regulate vertebrate development. Specifically, this is approached by studying a small number of selected genes which we believe have important regulatory roles. These are Dlx-3, a vertebrate homolog of the Drosophila homeodomain gene Distal-less, Pagliaccio and TCK, which are members of the Eph class of receptor tyrosine kinases and are expressed in unique patterns in the early Xenopus embryo, and Ventral Neural Cadherin (VN-Cad), a calcium-dependent cell adhesion molecule expressed in the ventral neural tube of zebrafish embryos.

The developmental functions of these genes are investigated by introducing experimentally regulated or constitutively active expression in specific tissues of transgenic embryos. Disruption of normal function is also achieved by targeted deletion of the endogenous gene and by dominant negative gene expression or antisense methods. The effects of these treatments on the affected embryos is assessed at the tissue, cell and biochemical levels.

Methods Employed:

An extensive array of molecular cloning techniques is used to generate and modify the genomic and cDNA clones used in this study. Micro-injection of RNA into cleaving blastomeres of *Xenopus* or Zebrafish, and transgenic mice are the primary functional assays for regulatory elements. The function of specific protein molecules are tested by injecting synthetic mRNAs that have been engineered to disrupt the signal transduction pathways used by the products of the endogenous gene. The effects this has on development and on embryonic cell-cell communication is monitored by *In situ* hybridization, immunocytochemistry, and reverse-transcription PCR procedures. Developmental functions are also revealed by ectopic expression of regulatory genes such as Distal-less in inappropriate tissues, which can result in disrupted tissue differentiation. DNA-protein interactions are evaluated by gel mobility shift and footprinting assays. Functional aspects of transcription factors are tested by co-transfection of expression and reporter constructs into tissue culture cells, and by *in vitro* transcription. Classical embryological techniques such as microdissection, cell dissociation, and tissue recombination are also used. Protein-protein interactions are identified by two-hybrid screening in yeast and by co-immunoprecipitation assays. Tyrosine phosphorylation is detected by western blots with antiphosphotyrosine antibodies, usually following immunoprecipitation.

Major Findings:

Ectopic Dlx3 expression in basal keratinocytes alters the identity of these cells, partially transforming them into more differentiated keratinocytes. Phenotypic changes include activation of the profilaggrin and loricrin genes, termination of cell division, reduction in hair follicle density and disruption of the epidermal barrier to water loss.

The embryonic dissociation elicited by activated Pagliaccio can be rescued by administration of additional cadherin to the cells by RNA microinjection.

Activation of Pagliaccio interferes with gastrulation movements in ectodermal explants induced with activin.

Significance to Biomedical Research and the Program of the Institute:
Xenopus, mouse and zebrafish embryos are versatile and complementary models for the study of vertebrate development. Each system has unique attributes that facilitate certain experimental approaches; for example, gene transfer into the mouse is an excellent method for testing the function of developmentally significant molecules, and the Xenopus embryo is ideally suited for studying cell-cell interactions during development. The zebrafish is highly amenable to broadly based mutagenic screens, and can also be physically manipulated in the same was as Xenopus. Together these three systems make a powerful combination that can be used to identify basic and phylogenetically conserved mechanisms controlling vertebrate, and thus human, embryogenesis and tissue differentiation. The studies carried out in this project should help elucidate molecular mechanisms regulating skin development and homeostasis, nervous system pattern formation and other developmental processes. Insights gained into the control of cell-cell adhesion should also be relevant to the invasive behavior of metastatic neoplasia. These are all relevant to human health and are areas of major importance to the mission of NICHD.

Proposed course of project:

Dlx3
The primary focus relating to Dlx3 will be determining the precise function of this gene in epidermal differentiation. We have shown by mis-expression experiments that Dlx3 can autonomously elicit a partial transformation of basal keratinocytes into a more differentiated state, as judged by appropriate molecular markers. This strongly suggests that Dlx3 could regulate genes in the epidermis that are part of the normal program of skin development. One way to test this hypothesis is to disrupt Dlx3 by targeted gene inactivation. Last November, we prepared two independent isolates of ES cells in which one copy of Dlx3 has been replaced with a G418 resistance cassette. Recently, these lines have been used by our collaborators, K. Mahon and A. Grinberg, to generate chimeric mice. We will soon be in a position to cross heterozygous animals. Dlx3-null

embryos will be analyzed at the gross, histological and biochemical levels, with particular attention to skin morphology and differentiation.

All of the Dlx family members are expressed in cranial neural crest that gives rise to facial features. Disruption of Dlx2 results in craniofacial abnormalities (Qiu et al., Genes Dev 1995, vol 9 p 2523), and it is possible that loss of Dlx3 function will have a similar phenotype. This will be evaluated by gross morphological criteria (e.g. cleft palate) and by examination of serial sections of mouse embryos, focusing on the head region. This will be carried out by Dr. Mahon's laboratory at Baylor. Since Dlx3 is not expressed in the central nervous system, brain/spinal cord defects are not expected. The primary effort in our laboratory will be on epidermis, particularly the orderly differentiation of basal cells into spinous and granular layer keratinocytes. This will also be evaluated by both gross skin morphology and by histological analysis using thin sections of epidermis from Dlx3 null embryos, neonates and adult mice, if the animals survive. We will test for effects on the expression of genes normally activated in the granular cell layer, such as profilaggrin and loricrin, by in situ hybridization, immunocytochemistry, and northern analysis.

In view of other Dlx knockout phenotypes, it is unlikely that the Dlx3 knockout will prove to be an early developmental lethal. However, if this turns out to be the case, it will be apparent from absence of the homozygous state in offspring, which will be monitored by routine genotyping. Examination of early embryonic stages will be carried out, using standard approaches. If we observe no alterations in the epidermal phenotype resulting from Dlx3 gene inactivation, we will consider the possibility of a combined knockout of Dlx3 and Dlx5, which is also expressed in epidermis. This would be a major undertaking, and would of necessity be a collaborative effort with another laboratory.

We will also continue to study the effects of Dlx3 expression on basal keratinocytes. We have shown that two structural genes, loricrin and filaggrin, are prematurely activated in these cells by Dlx3, and that Dlx3 protein binds in vitro to the filaggrin gene promoter. We will continue to investigate the role of Dlx3 in regulating filaggrin gene regulation.

Using the SELEX procedure for PCR amplification of protein-binding DNA elements we have determined that the optimal DNA binding site Dlx3 is ATAATTG(C/G). We have cloned this binding site , and an appropriate mutant oligonucleotide (verified by mobility shift competition analysis), in three tandem copies upstream from a basal promoter (HSV TK promoter, -50 to +50) driving the bacterial CAT reporter gene. We have also cloned the Xenopus Dlx3 coding sequence in an expression plasmid. The reporters and Dlx3 expression plasmids will be co-transfected at a range of DNA ratios into mammalian HeLa (Dlx3-negative) and NHEK (Dlx3-positive keratinocyte) cells. The expression of the reporters will be measured by CAT assays. Preliminary results indicate that Dlx3 may act as a repressor in this context. This is somewhat surprising given the presence of a Dlx3 binding site in the profilaggrin promoter, which is activated, not repressed, in basal cells mis-expressing Dlx3. However, if Dlx3 is indeed a repressor, then the activation of suprabasal genes we see in basal cells expressing Dlx3 may imply an interaction between Dlx3 and one or more co-factors that could modulate its impact on target gene transcription. Alternatively, the effect of Dlx3 on profilaggrin expression could involve an indirect mechanism, possibly involving one or more intermediate transcriptional activators or repressors that participate in the regulation by Dlx3. An important future aim of this project will be identification of these other proteins.

We also plan to use differential display or subtractive hybridization techniques to search for additional Dlx3 target genes.

Our observation that ectopic expression of Dlx3 in basal cells results in profilaggrin and loricrin gene transcription indicates that part of the program of gene expression of granular cell keratinocytes has been activated. We do not know what other suprabasal genes are affected, and therefore potential targets for Dlx3 action. Furthermore, it is possible, as noted above, that Dlx3 functions by activating or inactivating the expression of intermediate transcription factors. To address these problems we will compare basal keratinocytes that express Dlx3 ectopically to normal basal keratinocytes that do not. Normal and expressing cells can be isolated from the epidermis of normal or affected neonatal mice, and cultured for up to a few weeks in reduced calcium medium. Suprabasal cells can be removed by differential adhesion to fibronectin-coated plates. The affected basal cells do not seem to dissociate from the basement membrane in situ, so we believe their adhesive properties have not been compromised, and this fractionation procedure should be effective. RNA isolated from purified cells will be used to generate cDNA for either a differential display analysis, or possibly for use in a PCR-based subtraction scheme. Similar methods have been used successfully to identify differentially expressed genes, for example in metamorphosing frog tissues. One problem with this approach is that it looks at skin cells at a point fairly far along in the differentiation of this tissue. The salient regulatory events might by transitory in nature and could occur shortly after the initial activation of Dlx3 expression. In this case we would miss such Dlx3 targets by using epidermal cells from neonates.

One possible solution to this problem that we are considering is based on use of an inducible chimeric version of Dlx3 fused to a glucocorticoid receptor polypeptide. Such a chimeric protein can be activated by exposure to appropriate ligands, enabling inducible Dlx3 expression. Such a construct could be introduced into transgenic mice using a skin-specific promoter, or alternatively into Xenopus by recently developed transgenic methodology. The Xenopus option has the potential attraction of avoiding the complication of exposure of the embryo in utero to steroid hormones. In either case, basal keratinocytes would be isolated from the epidermis of transgenic animals (neonatal mice or postmetamorphosis froglets), induced with steroid for variable times and RNA isolated for analysis. Such an approach would yield kinetic response data in addition to providing a source of additional genes under direct or indirect control of Dlx3.

Eph Class Receptor Tyrosine Kinases.

We will continue to focus on two members of this family; Pagliaccio (Pag), which is activated in mesoderm at early gastrula and is transiently expressed later in the developing nervous system and TCK (tail/cement gland kinase), which is present as a maternal RNA and becomes localized during early organogenesis.

We have shown that when activated in cleaving Xenopus embryos, Pag leads to a complete but fully reversible loss of cell adhesion. This effect can be rescued by injection of cadherin mRNA, suggesting that one of more of these calcium-dependent adhesion molecules could be the target for Pag action. This possibility will be investigated in embryos by testing the ability of other cell adhesion components, such as catenins, and mutated versions of cadherins to function in the rescue assay.

So far all of the cadherins tested, C-cadherin, E-cadherin and N-cadherin can rescue the dissociation phenotype elicited by Pag activation. This could be due to a straightforward increase in the adhesive properties of the cells, counteracting the disadhesion effect of Pag, or the introduced cadherins could be interacting with Pag in a stoichiometric manner, preventing interference with endogenous adhesion molecules. This can be tested by using modified cadherins in the rescue experiment. For example, injection of N- or E-cadherin lacking the catenin-binding elements at the C terminus would not directly increase adhesion, since these cadherins are unable to associate with the cytoskeleton. If such constructs lead to reversal of the Pag dissociation phenotype, the direct interaction model would be favored.

Likewise, other adhesion components, such as α-catenin or plakoglobin/β-catenin for which full-length recombinant clones exist can also be tried. Some of these have phenotypes of their own when injected, but these are not manifested at the cleavage stage when Pag-induced dissociation takes place.

In addition to the dissociation phenotype, we will analyze the ability of Pag to influence cell-cell interactions during and after gastrulation. We have already shown that activation of Pag can inhibit the gastrulation movements induced in ectodermal explants by activin treatment. This phenomenon will be further characterized by co-injection of lineage tracers. The role of Pag-regulated cell adhesion in establishing tissue boundaries (as appears to occur in rhombomeres, according to the Wilkinson and Holder laboratories) will be evaluated in embryos and in recombined embryonic explants by injecting RNA encoding EGFR-Pag chimeric molecules and a mutated TGFα that is not processed, and therefore remains anchored to the membrane. This should mimic the interaction between Pag and its natural ligand or ligands, which are membrane anchored. The advantage of this approach is that it does not interfere with endogenous Pag, ligands, or other members of either the Eph RTK or ligand families. Lineage tracers will be used to evaluate the extent to which these molecules promote (or prevent) the mixing of blastomeres at normal embryonic boundaries, or at artificial boundaries between recombined ectodermal explants.

Pag-cadherin interactions will also be examined in cultured mammalian cell lines in which adhesion depends exclusively on transfected cadherins.

Based on our embryological studies we hypothesize that Pag affects the function of C-cadherin, the major adhesion molecule in the early frog embryo. To test this idea we making use of a derivative of chinese hamster ovary (CHO) cells that have been transfected with a construct expressing Xenopus C-cadherin (gift of B. Gumbiner). These cells have an epithelial phenotype, dependent upon the proper adhesive function of the Xenopus cadherin. If our interpretation of the embryological experiments is correct, then activation of the Pag-EGFR chimera with EGF should interfere with this adhesion system and convert the cells to a more mesenchymal character, similar to the parental CHO cells that lack cadherin.

Following initial characterization, these lines will be treated with EGF to activate the Pag signalling pathway, and the effects monitored by several methods. Changes in the adhesive properties of the cells, or other relevant cellular behavior (e.g. migration) or morphology will be evaluated by microscopic examination of the cells on fibronectin-coated slides, at various plating densities. Particular attention will be paid to the pattern of cytoskeletal organization, which can be evaluated by staining with fluoresceinated phalloidin or antibodies to cytoskeletal proteins, and the integrity of adherens junctions, which can be tested either by immunohistochemistry or by co-immunoprecipitation of adherens junction proteins by cadherin antibodies.

If we observe an effect on adhesion, then this will provide an excellent system for future analysis of the cell biology of Pag function, and studies on the associated signal transduction pathways. If cell-cell adhesiveness of the cadherin-CHO cells does not respond to Pag activation, then the effects observed with Xenopus embryos may depend on adherens junctions components or cytoskeletal proteins that are not common to Xenopus blastomeres and mammalian cells.

Most of our efforts have been devoted to analysis of Pag, and this will continue to be the case for the time being. However, we are also interested in another Eph class RTK, called TCK. This gene is expressed in cement gland, midbrain, and in the somites and could be important in axon targeting to the cement gland and/or neural crest migration through sclerotomes in the somites.

Eventually we hope to test the function of TCK using a chimera approach similar to that which we have used for Pag.

In addition, in an attempt to identify novel targets for Pag, a yeast two-hybrid screen has been carried out using the Pag intracellular domain as "bait". Approximately 100 positive clones have been isolated and will be analyzed for possible interaction with Pag.

These clones will be screened by two criteria: A yeast mating assay will be carried out to identify any clones that are able to activate the selection gene (HIS3) in the absence of the Pag-Gal4 fusion. Such clones will be eliminated from further analysis. The second screen will be for production of beta galactosidase from the reporter gene, which depends on transactivation from the interacting hybrid molecules. Only clones generating beta galactosidase levels comparable to that of the control (p53-SV40 Tag) plasmid will be analyzed further. Beta galactosidase will be measured using a simple luciferase assay (Tropix, Inc). Clones passing both tests will be sequenced. Attention will focus on candidates that have appeared more than once in the screen, and appear to genuinely interact in the yeast context. The criteria that will be used to decide if a clone merits extensive further analysis are somewhat subjective; little is known about the signal transduction pathway used by Eph class receptors, so it is difficult to predict what kind of molecules to expect. Any adhesion or cytoskeleton-associated protein would be attractive, but unexpected or unidentifiable sequences will not be disregarded if they appear otherwise promising.

VN-Cadherin

A full-length VN-cadherin open reading frame has been recently obtained. This will be used to generate complete and truncated VN-cad RNA for injection into fish embryos, to test the hypothesis that this cadherin influences axon fasciculation and guidance.

This project is in its final phases, and will end when Jeff Franklin leaves the lab. Meanwhile, we will attempt to test the function of Vn-cadherin as follows: Full-length RNA encoding VN-cadherin will be injected into zebrafish embryos at the 2-4 cell stage, along with RNA encoding GFP to act as a lineage tracer. Axon patterns will be examined in surviving embryos by whole mount and thin section immunocytochemistry using antibody recognizing acetylated tubulin. We will perform preliminary examinations, and promising specimens will be further analyzed in a collaboration (initiated by Jeff Franklin) with Dr. Steve Easter at the University of Michigan. If, as hypothesized, VN cadherin plays a role in guidance or bundling of axons in the region of the embryo where it is expressed, then disturbance of axon morphology should result and should be detected by this method. Preliminary results indicate that this is the case.

VN-cad RNA will also be tested for the ability to rescue embryonic dissociation in Xenopus embryos injected with Pag constructs.

> This experiment is not expected to be very informative regarding the function of VN cadherin, but is intended rather to test the capability of Pag to interact with a Class 2 cadherin (so far all the types we have tested have been of the Class 1 "Classic" cadherin type).

Publications

Scales, J.B., Winning, R.S., Renaud, C.S., Shea, L.J. and Sargent, T.D. (1995) Novel members of the Eph receptor tyrosine kinase subfamily expressed during Xenopus development. Oncogene 11, 1745-1752.

Franklin, J.L. and Sargent, T.D. (1996) VN-Cad, a novel cadherin expressed in a subset of neural tissues in the zebrafish embryo. Dev. Dynamics 206:121-130

Winning, R.S., Scales, J.B. and Sargent, T.D. (1996) Disruption of cell adhesion in Xenopus embryos by kinase activity of Pagliaccio, and Eph-class receptor tyrosine kinase. Dev. Biol. In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01008-07 LMG

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Genetics of Protein-Nucleic Acid Interactions in Drosophilia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Susan R. Haynes	Expert/Senior Staff Fellow	(All listed LMG:NICHD)
Others:	Cecilia Pazman	Visiting Fellow	
	David T. Stolow	IRTA Fellow	
	Christine Shunk	Biologist	
	Hongtao Ma	NRC Research Associate	

COOPERATING UNITS *(if any)*

Ann Beyer, Department of Microbiology, University of Virginia Medical School

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Unit on Protein-Nucleic Acid Interactions

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.7	3.6	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to understand the roles of RNA binding proteins in the post-transcriptional regulation of gene expression, through an analysis of the in vivo functions of proteins that contain RNA recognition motif (RRM) domains. RRM domains have been identified in many proteins involved in post-transcriptional regulation, and can mediate sequence-specific binding to RNA targets. We use genetics, biochemistry and molecular biology to study six Drosophila RRM proteins. Three of these proteins, HRB87F, HRB98DE and CAZ, are homologs of vertebrate proteins that bind nuclear pre-mRNA (hnRNP proteins). We have isolated mutations in both Hrb genes. The mutation in the Hrb87F gene completely deletes the coding region. Homozygous mutant flies are viable and fertile, demonstrating that the HRB87F protein is not essential. However, in the appropriate genetic background, these flies are sensitive to the dosage of HRB98DE. Using this observation, we performed a genetic screen and isolated a mutation in Hrb98DE. This mutation abolishes the expression of the protein, as shown by Western blots of protein extracts from adults. Homozygous or hemizygous individuals show somewhat reduced viability but, like HRB87F, HRB98DE is not essential. However, homozygosity for both mutations is lethal, demonstrating that flies require a minimal level of HRB proteins for survival and suggesting that the two HRB proteins are functionally redundant. In vitro studies have suggested that hnRNP A1, a vertebrate homolog of the HRBs, may be a general splicing regulator. Data from overexpression and reduced expression fails to support such a role for the HRB proteins. However, preliminary data suggest that in the appropriate sensitized genetic backgrounds, reduced Hrb gene dosage can modulate the expression of the Ultrabithorax gene, which regulates segment identity. The CAZ protein, the Drosophila homolog of hnRNP P2, shows widespread but not ubiquitous expression during development. We have identified a novel RRM-containing protein that interacts with the CAZ protein in a yeast two-hybrid selection screen, and that may be a specific partner in ribonucleoprotein complexes. We have also generated deletion mutants and transgenic fly strains for use in mutagenesis screens for caz mutants.

PHS 6040 (Rev. 5/92)

Project Description

Personnel:

S. Haynes	Expert/Senior Staff Fellow	LMG, NICHD
H. Ma	NRC Fellow	LMG, NICHD
C. Pazman	Visiting Fellow	LMG, NICHD
C. Shunk	Biologist	LMG, NICHD
D. Stolow	NRC/IRTA Fellow	LMG, NICHD

Objectives:

Despite major advances in recent years, there are still many unresolved questions regarding the mechanisms of gene expression and regulation, particularly regarding the involvement of post-transcriptional processes in gene regulation. Understanding these processes will require a detailed knowledge of the protein-nucleic acid interactions that are involved. We are interested in analyzing the in vivo roles of RNA binding proteins in post-transcriptional regulation. The fruit fly *Drosophila* is a useful organism for such studies, since it offers the opportunity to apply genetics as well as biochemistry and molecular biology to the study of these processes in a multicellular eukaryote.

Methods Employed:

Culture and genetic analysis of *Drosophila* are done by standard methods. Creation of transgenic fly strains follows established procedures. Genes are localized by in situ hybridization to polytene chromosomes and by hybridization of isolated DNA fragments to Southern blots of genomic DNA from deletion chromosomes. Analysis of gene expression within developing embryos follows protocols developed by Tautz for use of digoxigenin-labeled probes for whole mount in situ hybridizations. Standard recombinant DNA technology is employed to screen phage cDNA and genomic DNA libraries for clones of interest, and to isolate, subclone, restriction map and sequence the DNA. Demonstration of RNA binding activity is done by UV cross-linking and gel shift analysis. Identification of RNA targets of RNA binding proteins is done by in vitro selection assays (SELEX) using in vitro transcription and PCR amplification of oligonucleotides that bind with high affinity. Preparation and analysis of RNA from different stages of development follows standard procedures. Antibodies directed against fusion proteins or peptides are prepared by standard means. Proteins are analyzed on Western blots of one-and two-dimensional gels. Yeast two-hybrid systems are used to identify interacting proteins.

Major findings:

In the cell, RNA is always associated with proteins, which are involved in its packaging, processing, transport, stability and translation. Many of the proteins involved in these processes have a common, well-conserved RNA binding domain termed the RNA recognition motif (RRM). Our studies have focussed on several RRM proteins. Three of these proteins, HRB87F, HRB98DE and CAZ, are components of *Drosophila* hnRNP complexes, which consist of newly synthesized pre-mRNA associated with specific nuclear proteins. These complexes and their component proteins have been extensively characterized in vertebrates, but the functions of individual proteins are largely unknown. The *Drosophila* HRB proteins have two copies of the RRM and a glycine-rich C-terminal domain, and are very similar to vertebrate hnRNP A1 and related proteins. The CAZ protein, which is the *Drosophila* homolog of hnRNP P2, contains an atypical RRM, a zinc finger-like sequence, and three glycine-rich domains. To assess the in vivo functions of these pre-mRNA binding proteins, we are generating mutations in the genes and analyzing their phenotypic effects. Two other proteins studied in the lab, RB97D and TSR, contain RRMs that are closely related to those of the HRB proteins. Both proteins are required for normal male fertility, and their roles in spermatogenesis are being assessed by biochemical and molecular genetic approaches.

Hrb87F and *Hrb98DE.* We have previously reported the results of genetic analysis of *Hrb87F*, and briefly summarize the data relevant to this report. *Hrb87F* is not essential; flies homozygous for a deletion of the coding region are viable and fertile. However, when this deletion is combined with a large deletion removing one copy of the *Hrb98DE* gene, males flies show significantly reduced fertility. Furthermore, in the presence of a mutation in the *Tsr* RRM protein gene, these flies are completely sterile. These effects on fertility can be wholly or partially rescued by a transgenic copy of *Hrb98DE*, demonstrating that hemizygosity for *Hrb98DE* contributes to the phenotype. This observation was used as the basis for a genetic screen for mutations that interact to cause male sterility or reduced fertility. We expected that this screen should generate mutations in *Tsr*, and possibly *Hrb87F* or *Hrb98DE*. A small-scale pilot test of this mutagenesis scheme did yield a mutation in *Hrb98DE.* [Note that we and Ann Beyer's lab had previously attempted to isolate *Hrb98DE* mutations directly, by screening for mutations that were lethal over a deletion of the locus, but no *Hrb98DE* mutants were isolated.]

The sterility phenotype of the new mutant could be rescued by a transgenic copy of *Hrb98DE*, and the mutation mapped genetically to the region of the *Hrb98DE* locus; accordingly, it has been named $Hrb98DE^1$. Homozygous $Hrb98DE^1$ flies are fertile, and show slightly reduced viability when compared to their heterozygous sibs. In fact, these flies can be maintained as a homozygous line. Thus, neither *Hrb* gene is essential for viability. However, homozygosity for mutations in both *Hrb* genes (a Hrb^- genotype) is lethal. This indicates that flies require at least a minimal level of HRB proteins for survival. Hrb^- individuals die as embryos or larvae, and examination of the dead animals reveals a variety of defects, including abnormal cuticular structures and head development, and holes in the cuticle. The variability in defects may reflect differences in when individual animals exhaust the supply of HRB proteins synthesized from maternal *Hrb* mRNAs that are deposited in the egg during oogenesis.

Further evidence that the mutation we have isolated is in the *Hrb98DE* gene comes from

Western blot experiments, using an antibody directed against the RRMs. Wild-type adult females express high levels of HRB98DE, but the protein is undetectable in extracts from homozygous mutant females. We can detect transcripts by RT-PCR, however, indicating that the mutation does not abolish transcription of the gene. We are currently sequencing the mutant gene to identify the specific lesion.

Human hnRNP A1 has been proposed to be a general splicing factor, based on its ability to counteract the effects of other splicing factors in promoting specific alternative splice site choices. However, experiments with overexpression and reduced expression of the HRB proteins do not support such a general role for the HRB proteins. To date, splicing of only one *Drosophila* mRNA (from the *Dopa decarboxylase* locus) has been shown to be affected by overexpression of HRB proteins. Overexpression causes an aberrant splice site choice, but the effect is transient, despite continued high levels of HRB protein. Normal splicing of this mRNA is not dependent on HRB proteins. We have begun experiments to identify mRNA whose processing in vivo is dependent on HRB proteins. HRB protein expression may be most critical for processing transcripts with large introns or complicated alternative splicing pathways, such as the *Ultrabithorax* (*Ubx*) gene. *Ubx* has the added advantage that differences in expression levels give distinguishable phenotypic differences. Reduced UBX expression gives rise to a partial homeotic transformation of haltere to wing. Preliminary experiments demonstrate that, in a sensitized genetic background, the level of UBX expression (judged phenotypically) is dependent on the HRB proteins. The test flies are heterozygous for both a *Ubx* mutation and a second-site mutation that enhances the *Ubx* phenotype. Mutations in the *Hrb* genes partially suppress the enhancement, suggesting that they interact with either *Ubx* or the second-site enhancer mutation.

caz. The *caz* gene is located on the X chromosome, about 10 kb away from the *easily shocked* gene. We are using several complementary approaches to analyze its function. We have begun several mutagenesis screens for *caz* mutants. A viable P element insertion in *easily shocked* has been mobilized by P transposase to generate deletions and transpositions into nearby genes. Although some deletions were obtained, none extended into *caz*. These will be useful in other screens, however. New transpositions are being assayed by a combination of plasmid rescue, PCR and southern blotting. To date, no *caz* insertions have been detected. Another screen involves chemical mutagenesis by EMS, and should be unbiased in terms of target susceptibility. We are screening for mutations that are lethal over a deletion that includes *caz* and several adjacent genes. Putative *caz* mutants will be localized by further deletion mapping. To provide positive identification of *caz* mutants, we have established a transformant line carrying a *caz* cDNA construct.

Many RRM proteins function as components of multiprotein complexes. To identify proteins that interact with CAZ, a yeast two-hybrid screen was performed, and one interacting protein was identified. This protein is related to an uncharacterized mouse protein that contains an RRM, and is potentially another RNA binding protein. The interacting portions of the two proteins map to the non-RRM regions.

Tsr. The *Tsr* gene encodes a cytoplasmic RRM protein that is expressed exclusively in the testis. We have previously reported the phenotypic characterization and protein expression pattern of *Tsr*. In the absence of this protein, most sperm fail to complete development, and instead degenerate in the testis. Our efforts during the past year have been focussed on identifying the biochemical role of TSR during spermatogenesis. Post-transcriptional regulation is very important during spermatogenesis, since most transcripts required after meiosis are synthesized premeiotically and stored as RNP complexes. Based on the timing of expression and subcellular localization of the protein, TSR could be involved in regulating mRNA stability/turnover or translatability during spermatogenesis. Possible roles and time of action were investigated using two-dimensional protein gel electrophoresis. If TSR acts solely post-meiotically, then lack of TSR should not affect the proteins synthesized before meiosis. If TSR has a pre-meiotic function, then the protein profiles of the pre-meiotic primary spermatocytes may be affected. We made use of the *bol* mutation to cause spermatogenesis to arrest before meiosis, so that the testes of adult males were filled with primary spermatocytes. Protein extracts were prepared from testes of wild type adult males and males with the following genotypes: *bol, bol* Tsr^-, and *bol* Tsr^- with a Tsr^+ transgene. Although most of the proteins were identical in the extracts from all the *bol*-containing males, there was an abundant protein detected only in males also lacking TSR. This protein disappeared in the presence of the Tsr^+ transgene, and was a normal component of protein extracts from wild-type testes. The results of this experiment indicate that TSR has a pre-meiotic function, and are most compatible with the idea that TSR acts to repress the expression of one or a few proteins before meiosis.

We have begun attempts to purify TSR-containing complexes from flies. Extracts from whole male flies were separated on sucrose gradients, and the TSR-containing fractions were identified by Western blotting. TSR migrates as a complex, and its distribution partially overlaps that of polysomes. Further experiments are in progress to determine whether TSR associates or merely comigrates with polysomes. We have recently developed procedures to immunoprecipitate TSR efficiently from protein extracts. This material will be used to identify other protein and RNA components of TSR complexes.

We reported previously that the ADP-ribosylation factor, ARF1, specifically interacts with TSR in a yeast two-hybrid interaction screen. To assess the biological relevance of this biochemical interaction, we are looking for genetic interactions between ARF1 and TSR. There are no known mutants of *Arf1*, and no deletions that remove the gene. However, we have identified a P element insertion nearby. Initial experiments to generate deletions extending into the *Arf1* gene have not been successful. However, we have recently begun a new screen, based on techniques developed in the Engels lab, that is likely to yield the desired mutant.

Rb97D. The *Rb97D* gene encodes an RRM-containing protein that is expressed in the nuclei of primary spermatocytes and is required for spermatogenesis. During spermatocyte development, three regions of the Y chromosome become transcriptionally active and cytologically visible as large lampbrush loops. We have previously reported that RB97D binds to a specific lampbrush loop, the C loop. We are using two approaches to identify the RNA targets for RB97D. The lampbrush loop transcripts arise from a region containing satellite sequences and transposable elements; protein-coding sequences within the loop are postulated to exist, but have not yet been identified. Other researchers have identified the major families of transposable elements found in the C loop. If RB97D binds to transposable element transcripts, it might affect the expression of those transposable elements transcripts when they are located in other genes. We have tested this hypothesis genetically, using strains in which transposable element insertions give a visible phenotype. To date, we have found no transposable elements whose expression is modulated by mutations in *Rb97D*. Our second approach is to identify poly(A)$^+$ RNAs derived from the C loop, and analyze whether their expression is affected by mutations in *Rb97D*. This approach relies on PCR-based amplification strategies to identify transcripts that differ between two RNA samples. We have generated fly strains that contain four or no copies of the C loop region, and are preparing poly(A)$^+$ RNA from the two strains to use in these experiments.

Significance to Biomedical Research and the Program of the Institute:

A major aim of the Institute research program is an understanding of the molecular mechanisms of the regulation of gene expression. RNA binding proteins play critical roles in the metabolism of RNA transcripts. *Drosophila*, because of the applicability of genetic, molecular and developmental techniques in this organism, offers significant advantages in the study of such problems. The identification of RRM-containing proteins that regulate alternative splicing in the *Drosophila* sex determination pathway, and biochemical evidence for the involvement of mammalian hnRNP proteins in splicing, have stimulated interest in the study of such proteins as important regulatory molecules.

Proposed course:

Hrb87F and Hrb98DE. We will continue to analyze the mutant phenotypes of the *Hrb98DE*^{1} allele and of the *Hrb87F-Hrb98DE* double mutant. We will perform detailed phenotypic analyses of dead embryos and larvae to identify specific developmental processes and genes that depend on HRB protein expression. We will examine the effects on *Ubx* expression in more detail, using antibody staining and RT-PCR assays to determine the molecular basis for the observed phenotypic suppression. We will also test other large genes with complex splicing patterns for dependence on HRB protein expression. These tests will involve constructing the appropriate fly strains to look for genetic interactions, then using molecular tools to identify the nature of any interactions observed. Previous observations suggest a potential involvement of HRB proteins in nervous system development and heat shock response. These will be tested by examining nervous system development in mutant embryos using monoclonal antibodies to neuronal proteins, and by examining the susceptibility of mutant flies to heat shock.

caz. A major effort will be directed toward generating a *caz* mutant and analyzing its phenotype. Depending on the results of these experiments, we will look for specific genes whose expression may be sensitive to the presence of CAZ. The *caz* mutant will also be used to assess the biological relevance of the interaction with the novel RRM protein detected by yeast two-hybrid assays. To begin a structure-function analysis of CAZ, we are generating *caz* transgenes with specific mutations in the RRM and the zinc finger-like region. These are under the control of a Gal4 inducible promoter which can be expressed at specific times in specific tissues after crossing to the appropriate inducer strains. These transgenes will allow us to examine the effects of overexpression of mutant CAZ protein, and to determine whether the mutant protein will rescue a chromosomal *caz* mutation. In addition, these transgenes and a wild-type CAZ transgene carry an epitope tag that will aid in affinity purification of CAZ-containing complexes.

Tsr. The efforts to generate *Arf1* mutants will be continued, and the mutants will be used to examine potential genetic interactions between ARF and TSR. We will continue our sedimentation analysis of extracts to determine whether TSR is present in polysomal mRNP complexes or in free mRNP. The immunoprecipitation procedure will be optimized to increase the yield without requiring laborious dissection procedures that might lead to degradation of TSR-containing complexes. If we can immunoprecipitate complexes in sufficient yield, we will look for additional proteins that might be coimmunoprecipitates (such as ARF), and attempt to amplify and clone RNAs that may be present in the complex. Any such RNAs will be analyzed biochemically by methods such as electrophoretic mobility shift analysis and UV cross-linking to determine whether they bind TSR.

Rb97D. We will prepare poly(A)$^+$ RNA from mutant males containing 0 or 4 copies of C loop sequences, and use it in differential display and representational difference analyses to detect transcripts differ between the two strain. These can be rapidly checked by Southern blotting to determine whether they are transcribed from the C loop. Candidate transcripts will be tested for specific high-affinity binding to RB97D, and for alterations in structure or expression in RB97D$^-$ mutant males.

Publications:

Haynes SR. Posttranscriptional regulation and RNA binding proteins in development. J Biomed Sci 1995;2:293-301.

Zu K, Sikes ML, Haynes SR, Beyer AL. Altered levels of the *Drosophila* HRB87F/hrp36 hnRNP protein have limited effects on alternative splicing in vivo. Molec Biol Cell 1996;7:1059-73.

LMGD

LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT

Heiner Westphal, M.D., Chief

The Laboratory generates gene-altered mice to study the molecular genetics of genes that control pattern formation in the embryo, T cell development and genomic imprinting. These mutant mice also serve as paradigms of human diseases.

MOLECULAR GENETICS OF MOUSE DEVELOPMENT

Genes that regulate vertebrate development are the topic of current and projected research in the Section on Transgene Regulation, directed by ***Heiner Westphal***. His group studies an exciting phase in mouse embryonic development when precursor cells receive signals that channel them toward regional specificity and differentiation. *Sonic Hedgehog* knockout mutants, the topic of one of the projects, exemplify the profound consequences of a disruption in a chain of commands that sets off this process. Likewise, the *Lhx3/4* double knockout mice are proof for the fundamental importance of transcriptional regulators in mediating the change from a committed to a specialized cell. The projects illuminate the actions of genes that are of fundamental importance for the development of the central and peripheral nervous systems, the kidney, and the pituitary gland. While the complexity of neuronal development is enigmatic and predicts deliberate progress, the studies of the embryonic kidney and adenohypophysis are expedited by a wealth of information available on cell specialization from common precursors present in the anlagen of these organs. Several sets of experiments elucidate the roles of LIM-homeodomain proteins and peptides that bind to them, of *Sonic Hedgehog* and of glia cell line-derived neurotrophic factor (GDNF) during embryogenesis. In each case, published and present work of the group has generated a solid base for future experimentation.

Sonic Hedgehog (Shh). This gene plays important roles in ventral patterning along the anterior-posterior axis of the developing central nervous system and in influencing pattern of cell types elsewhere in the developing vertebrate embryo. In collaboration with the laboratory of Philip Beachy at Johns Hopkins University, the Westphal group has analyzed the consequences of loss of Shh function in the mouse. Mutant embryos fail to establish or maintain midline structures such as notochord and floor plate. Patterning of the brain and the eyes is profoundly deficient, reminiscent of human holoprosencephaly. Major skeletal defects include the absence of distal limb bones, vertebrae and a rib cage. The Westphal laboratory currently evaluates the role of Shh in lung morphogenesis, its contribution to cell differentiation in sympatho-adrenal lineages, as well as the identity of Shh effector molecules.

LIM-homeobox (Lhx) genes. This family of developmental transcription factors regulates important steps of organogenesis. *Lhx2* and *Lhx7*, a pair of closely related genes, are essential for pattern formation, particularly in the developing central nervous system (CNS). Knockout analyses of these two genes are in progress to address their combinatorial functions in the CNS and elsewhere in the developing embryo. *Lhx3* and *Lhx4*, another closely related pair of genes, is responsible for the formation of the adenohypophysis, and also plays an essential role in motoneuron innervation along the spinal chord, a function currently being assessed via double loss-of-function analysis. In a pursuit of the mechanism of action of the *Lhx* genes, the Section has isolated Ldb1, a novel factor that binds LIM motifs present in LIM-homeodomain and LMO proteins. In collaboration with the neighboring laboratory of Igor Dawid, the group has shown that the murine Ldb1 and its frog counterpart XLdb1 synergize with vertebrate LIM-homeobox genes to induce early determinators of development and to form secondary axes when injected in frog embryos. The analysis of Lhx2 and Lhx7 functions in forebrain development continues. Current work on Lhx3 and Lhx4 is focussed on the role of these factors in the development of the adenohypophysis, and in conveying motor neuron identity. Also, the possibility of mutations in these genes as a cause of specific human genetic disorders is being evaluated.

Glial cell line-derived neurotrophic factor (GDNF) was isolated as a neurotrophic factor for midbrain dopaminergic neurons. In view of its neurotrophic activity on a wide range of neuronal populations in vitro and in vivo, GDNF is being considered as a potential therapeutic agent for neuronal disorders. During mammalian development, GDNF is expressed not only in the nervous system, but also very prominently in the metanephric kidney and in the gastrointestinal tract, suggesting possible functions during organogenesis. The Westphal group has investigated the role of GDNF during development by generating a null mutation in the murine GDNF locus. Aided by research groups in Denver and in Helsinki, the Section was able to demonstrate that mutant mice show kidney agenesis or dysgenesis and defective enteric innervation. GDNF induces ureter bud formation and branching during metanephros development. In addition, the factor is essential for proper innervation of the gastrointestinal tract. The Westphal group presently examines the role of GDNF in the development of the enteric nervous system and, in this context, attempts to rescue enteric innervation in embryonic gut explants that lack GDNF function. A role of the GDNF locus in Hirschsprung disease is being assessed as well.

COLLABORATIVE STUDIES

The Embryo Microinjection Facility, an integral part of the section, is a barrier mouse facility that houses up to 3000 mice and contains a centrally located animal manipulation and embryo microinjection laboratoryin which gene-altered mice are generated. Groups outside of the Westphal laboratory often request assistance in the design of their own experiments in this regard. As a result, the Section on Transgene Regulation becomes acquainted with new and exciting research avenues, especially those located at the interface between molecular genetics and medicine. This has had a very positive influence on the group's own research concepts and has helped them to attract a number of excellent coworkers that have been trained both as physicians and as molecular biologists. Listed here are only projects that were performed in collaboration with research teams outside NICHD. Collaborations with colleagues at NICHD may be found elsewhere in this volume (see the reports by Janice Chou and Domenico Accili).

Mouse models for Tay-Sachs and Sandhoff diseases. These human hereditary disorders result from mutations in the HEXA gene-(Tay-Sachs) or HEXB gene (Sandhoff) encoding the α and ß subunits, respectively, of the lysosomal enzyme ß-hexosaminidase A. As a result of these gene defects, excessive amounts of gangliosides accumulate in the CNS of the affected infants, and rapid motor and mental retardation results in early death. Richard L. Proia, GBB, NIDDK, requested the assistance of the Westphal laboratory in generating mouse models of these disorders. Via disruption of the HEXA and HEXB genes, respectively, mice were obtained whose enzyme defects mimicked those of the Tay-Sachs and Sandhoff patients, respectively, and who accumulated gangliosides in neuronal lysosomes in an age-dependent manner. The Tay-Sachs model showed no neurological abnormalities. By contrast, the Sandhoff model was severely affected. At about three months of age, the mice began to show progressive deterioration of motor function with gait abnormalities and spastic limb movement. At later stages severe muscle atrophy caused further motor dysfunction, and the animals were sacrificed. The phenotypic difference between these two mouse models is the result of differences in the ganglioside degradation pathway between mouse and man. The studies have uncovered some of the promises of mouse models that mimic human single-gene disorders. Especially the Sandhoff model is ideally suited for studies on the efficacy of functional enzyme replacement and/or gene therapy.

Mice that lack the sperm receptor Zp3. The laboratory of Jurrien Dean (NIDDK, NIH) has a long-term interest in the zona pellucida that surrounds the egg and the pre-implantation embryo. Of particular interest to his laboratory is the binding of sperm to the zona pellucida that induces the acrosome reaction and is required for zona penetration. The zona is composed of three sulfated glycoproteins, ZP1, ZP2 and ZP3. *In vitro* experiments have suggested that ZP3 is the primary sperm receptor. The Westphal group has collaborated with the Dean laboratory to disrupt the production of ZP3 by introducing an insertional mutation into the Zp3 gene of embryonic stem cells. These cells have been used to establish a Zp3 mutant mouse strain. In females lacking ZP3 follicles grow but the zona pellucida is absent and no offspring are produced. Biosynthesis of the other zona proteins was unaffected. This suggests that the assembly of the zona requires the presence of all three ZP proteins. Clearly, the results identify an intact zona as a prerequisite for fertility.

T CELL DEVELOPMENT

Research in the Unit on Cellular and Developmental Biology, led by ***Paul Love***, is directed at understanding the cellular and genetic events that control normal T cell development. Current studies focus on the role in thymocyte maturation of signals transduced by cell surface receptors, particularly the T cell antigen receptor (TCR). In mature T cells, the TCR complex is centrally involved in antigen recognition, T cell activation and cell-mediated immunity. In developing thymocytes, TCR signals are important for maturation and thymic (positive and negative) selection. How the TCR is capable of controlling these various processes remains a central question in immunology and is relevant to understanding human immunodeficiency and autoimmune disorders.

Role of TCR signaling molecules in T cell development. The main focus of current research in the laboratory is the elucidation of the role of T cell antigen receptor (TCR) signal transduction in thymocyte development. Recent work has been aimed at systematically dissecting and characterizing the function of the multiple signal transducing sequences contained within the TCR. TCR signal transduction sequences (termed *I*mmunoreceptor *T*yrosine-based *A*ctivation *M*otifs;

ITAMs) are contained within four distinct subunits of the multimeric TCR complex (ζ, CD3-γ, -δ, -ϵ) and are triplicated in the ζ chain cytoplasmic domain. Di-tyrosine residues within ITAMs are phosphorylated upon TCR engagement and function to recruit signaling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell activation cascade. Although conserved, ITAM sequences are nonidentical, raising the possibility that the diverse developmental and functional responses that are controlled by the TCR may be regulated, in part, by distinct ITAMs.

Experiments performed in the Love laboratory have involved over-expression of various TCR signaling subunits in transgenic mice with the expectation that perturbations in T cell development, if observed, would provide insight into the function of these molecules. The results of these studies suggest that individual TCR subunits perform specialized functions at different points in development as components of both the Pre-TCR (a signaling complex expressed in early thymocytes that includes some but not all of the TCR subunits) and the TCR. In addition, these experiments indicate that regulated expression of TCR signaling subunits during development is essential for ensuring that the appropriate signals are delivered to thymocytes at specific stages of maturation.

To examine the developmental role of ζ chain, the major TCR signaling subunit, ζ-deficient ($\zeta^{-/-}$) mice were generated by gene targeting. The phenotype of these mice demonstrated a critical function for ζ chain in promoting TCR surface expression and T cell development. The importance of ζ-chain ITAM signals was examined by reconstituting TCR surface expression in $\zeta^{-/-}$ mice using transgenes that encode full-length ζ-chain or ζ-chain variants that lack one, two or all three ITAMs. Examination of T cell development in these mice revealed the striking finding that ζ-chain signals were not specifically required for T cell maturation provided that TCR surface expression was restored and the remaining (CD3) signaling chains were present in the TCR complex. Further investigation of these mice has shown that the ζ-ITAMs are required for efficient thymocyte positive selection (a developmental process that ensures maturation of self-educated T cells and prevents maturation of potentially auto-reactive T cells). These results identify a previously unrealized function for the multiple TCR-ITAMs in amplification of TCR signals during thymocyte selection. Similar approaches are currently being used to examine the role of other TCR signal transducing subunits (eg., CD3ϵ) in T cell development and selection.

Role of CD5 in T cell development and selection. Accumulating data indicate that another cell surface receptor, CD5, plays an important role in thymocyte selection by influencing signaling through the TCR. CD5 surface expression is regulated by TCR engagement and differs between individual thymocytes depending upon the affinity of their TCR for positively selecting ligands. To determine if the level of CD5 expression influences the outcome of thymocyte selection the laboratory generated transgenic mice that over-express CD5 throughout development. Over-expression of CD5 significantly impaired positive selection of some thymocytes (those that normally express low levels of CD5) but not others (those that normally express high levels of CD5). Collectively, these findings support a role for CD5 in modulating the TCR signal transduction response in thymocytes and thereby influencing the outcome of thymocyte selection. The ability of thymocytes to regulate their surface expression of CD5 suggests that multiple cell surface structures can influence the signals delivered to thymocytes. This potential for signal modulation may be particularly useful in generating the maximum possible TCR diversity in the mature T cell repertoire. A probable mechanism for this function is via the activation-induced binding of regulatory molecule(s) to ITAM-like tyrosine containing sequences within the CD5 cytoplasmic tail. To directly assess the importance of the CD5 cytoplasmic domain, transgenic mice that express a tail-less form of CD5 (mCD5) are being generated. Both the intact and mCD5 transgenes will be used to reconstitute CD5 surface expression in $CD5^{-/-}$ mice to determine if cytoplasmic sequences are required for its activity. Finally, the laboratory is attempting to identify molecules that interact with CD5 that may be involved in regulating signal transduction by the TCR.

Identification of novel genes involved in T cell development. The laboratory has recently initiated a search for novel genes that may be important for thymocyte maturation. Using a reverse-transcriptase PCR (RT-PCR)-based strategy that employs degenerate oligonucleotides specific for certain classes of proteins (including protein tyrosine kinases, protein tyrosine phosphatases and homeobox genes), RNA from early fetal (day 13) or adult thymus is being screened. Using this approach, Love and his colleagues have identified a novel nonreceptor protein tyrosine kinase gene expressed in early thymocytes (*txk*). Sequence analysis indicates that Txk belongs to the Tec family of protein tyrosine kinases. *Txk* is expressed in thymocytes as early as fetal day 13.5 and its expression continues throughout development; *txk* transcripts are present in peripheral T cells and in mast cell lines but are not detectable in B cell, macrophage/monocyte cell lines or in non-hematopoietic fetal or adult tissues. In both thymocytes and T cells *txk* transcripts are downregulated after activation by TCR cross-linking. Transgenic mice that over-express wild-type and dominant negative (kinase dead) forms of Txk have been generated to determine the possible role of this kinase in T cell development. Preliminary results indicate that Txk may be involved in regulating thymocyte positive selection. Members of the laboratory have also begun to identify potentially novel homeobox-containing genes in the early fetal thymus. Expression of homeobox-containing genes in the developing thymus has not been specifically explored. Two potentially novel sequences were obtained from fetal thymus. The presence of conserved regions within the putative homeobox domain strongly indicates that these sequences are contained within homeobox genes. These genes are thus potential candidates for involvement in thymus/thymocyte development.

GENOMIC IMPRINTING

The Unit on Genomic Imprinting, led by ***Karl Pfeifer***, searches for a molecular explanation of the fact that normal mammalian development requires the contribution of haploid genomes from both parents. This non-equivalence of the two genomes is a result of differential epigenetic modifications ("imprints") that occur in the germlines and that lead to unequal expression of the two parental alleles during development. To date a number of genes have been identified where expression is restricted to either the maternal or to the paternal allele. These imprinted genes map to discrete clusters in the mammalian genome. The group has begun to investigate the molecular mechanisms for this unusual form of gene regulation by focussing on the distal portion of mouse chromosome 7. This cluster contains at least five imprinted genes. The genes encoding p57KIP2, a cyclin-dependent protein kinase inhibitor, and Mash-2, a trophoblast-specific transcription factor, lie at the telomeric end of the cluster and are expressed only from the maternal chromosome. The two growth factor genes, *Ins-2* (insulin-2) and *Igf-2* (insulin-like growth factor-2) map to the middle of the cluster, both are paternally expressed. H19 lies at the centromeric end of the cluster and encodes a maternally expressed untranslated RNA. Disruptions in allele-specific expression at this cluster leads to embryonic lethality in the mouse and are correlated with Wilms tumor in humans.

Allele-restricted expression of the H19 gene. Experiments are aimed at determining the distinctive structures of the maternal and paternal chromosomes that allow them to be differentially recognized by the transcription machinery in the nucleus. The group has developed a transgenic model system to investigate genetic requirements for imprinting at the H19 locus: wild type transgenes are hypermethylated and silent when inherited through the paternal germline but are unmethylated and expressed when passed on through the maternal germline. Deletion analysis has identified two regions required for this allele-specific expression. Intriguingly, of these two is the H19 structural gene itself. Current studies focus on differentiating a role for this element as a *cis* acting DNA regulatory element or as a regulatory RNA encoding element. The second region required for imprinting includes DNA sequences in the 5' flank. These sequences are not required for normal expression of the transgene but only for restricting expression to the maternally inherited transgene. These sequences are also the targets of DNA methylation, *i.e.* this upstream element is preferentially methylated when inherited through the paternal germline. Using homologous recombination and embryonic stem cell technology, the unit is generating mice with mutations in both of these regions to confirm and extend the results observed with the H19 transgene. These results will also form the basis for developing a protocol for a mutagenesis screen to identify factors acting in trans to regulate H19 expression.

Why are imprinted genes clustered? Addressing this problem is a way of investigating the general role of genome organization mammalian biology. The H19 and *Igf-2* genes are separated by about 90 kb in both the murine and the human genomes. The temporal and spatial expression patterns of these two genes are strikingly coincident. The two genes, however, are reciprocally imprinted and therefore are not both expressed from the same chromosome. A mechanistic linkage to the expression patterns of these two genes has now been clearly established. First, the two genes have been shown to share a common set of enhancers and second, deletion of the H19 gene and upstream sequences on the maternal chromosome results in the inappropriate expression of the maternal allele of *Igf-2*. Dr. Tilghman and colleagues at Princeton University have proposed a model that explains the reciprocal imprinting of these genes as occurring because of a competition of the various promoters for a common set of enhancers. An alternate model posits that chromosome-specific activation of a boundary element (or insulator) is critical in determining allele-specific expression of *Igf-2*. The Pfeifer group has generated mice carrying a deletion in the upstream sequences that are required for imprinting but not for expression of the H19 transgene. Examination of the expression of *Igf-2* on mutant chromosomes will directly test the two models for reciprocal imprinting at this gene cluster. *Igf-2* and *Mash-2* were identified as imprinted genes fortuitously during studies investigating the phenotype of mice carrying genetically engineered disruptions of these loci. H19, *Ins-2*, and p57KIP2 were all identified and mapped independently and subsequently checked for allele-specific expression given their proximity to *Igf-2*. The unit has begun a systematic search for other genes in this chromosomal region. After constructing a physical map of the region, the group is presently identifying Bacterial Artificial Chromosome clones carrying inserts that span the entire distal chromosome 7 region. These probes will be used to develop a transcript map of the region. Novel genes will be assessed for allele-specific expression and for possible roles in the etiology of Wilms tumor and Beckwith-Wiedemann Syndrome.

LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT

Chief, Heiner Westphal, M.D.

Tenure -Track Investigators
Paul Love, M.D., Ph.D
Karl Pfeifer, Ph.D

Staff Scientist
Eric Lee, D.V.M.

Senior Fellows
Kathleen Mahon, Ph.D.
Takafumi Nakamura, M.D., Ph.D
Forbes Porter, M.D., Ph.D
Hui Sheng, Ph.D.

Guest Researchers
Chin Chiang, Ph.D.
Kenji Moriyama, M.D.
Jose Pichel, Ph.D.
Madulika Srivastava, Ph.D.
Naoyuki Takuma, M.D.
Yosef Yarden, Ph.D.
Chunfa Zhang, Ph.D.

Post-Doctoral Fellows
Hala Azzam, Ph.D.
Stefano Bertuzzi, Ph.D.
Ohad Birk, Ph.D.
Douglas Feltner, M.D.
Ying Litingtung, Ph.D.
Liya Shen, Ph.D.
Connie Sommers, Ph.D.
Tsuyoshi Yamashita, M.D., Ph.D.
Yangu Zhao, Ph.D.

Pre-Doctoral Fellows
Susan Baldwin
Salil Goorha
Todd Gould
Elizabeth Ives
Kin Lui
Chris Muntzel
Anne Pitts
Howard Shen
Andreas Tomac
Phi Wiegn

Senior Technical Specialists
Sing Ping Huang

ANNUAL BIBLIOGRAPHY

Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, José PA, Taylor SI, Westphal H. Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. Nature Genetics 1996;12:106-9.

Accili D, Fishburn CS, Drago J, Steiner H, Lachowicz JE, Park BH, Gauda EB, Lee EJ, Cool MH, Sibley DR, Gerfen CR, Westphal H, Fuchs S. A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. Proc Natl Acad Sci USA 1996;93:1945-9.

Albrecht FE, Drago J, Felder RA, Printz MP, Eisner GM, Sibley DR, Westphal H, Jose PA. Role of the D1A dopamine receptor in the pathogenesis of genetic hypertension. J Clin Invest 1996;97:2283-8.

Bertuzzi S, Sheng HZ, Copeland NG, Gilbert DJ, Jenkins NA, Taira M, Dawid IB, Westphal H. Molecular cloning, structure and chromosomal localization of the mouse LIM/homeobox gene Lhx5. Genomics 1996;36:234-9.

Combadiere, B, Freedman, M, Chen, L, Shores, EW, Love, PE, Lenardo, MJ. Qualitative and quantitative contributions of the T cell receptor ζ chain to mature T cell apoptosis. J Exp Med 1996;183:2109-17.

Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee EJ, Alt FW, Westphal H. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci USA 1996;93:5860-5.

Lei KJ, Chen H, Pan CJ, Ward JM, Mosinger B, Lee EJ, Westphal H, Mansfield BC, Chou JY. Glucose-6-phosphatase substrate transport in the glycogen storage disease type-1a mouse. Nature Genetics 1996;13:203-9.

Levine MS, Altemus KL, Cepeda C, Cromwell HC, Crawford C, Ariano MA, Drago J, Sibley DR, Westphal H. Modulatory actions of dopamine on N-methyl-d-aspartate receptor-mediated responses are altered in D1A deficient mutant mice. J Neurosci 1996;16:5870-82.

Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H. Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 1996;382:73-6.

Rankin T, Familiari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J. Mice homozygous for an insertional mutation in the Zp3 sperm receptor gene lack a zona pellucida and are infertile. Development 1996;122:2903-10.

Sango K, Yamanaka S, Hoffmann A, Okuda Y, Grinberg A, Westphal H, McDonald MP, Crawley JN, Sandhoff K, Suzuki K, Proia RL. Mouse models of Tay-Sachs and Sandhoff Diseases differ in neurologic phenotype, central nervous system pathology and ganglioside metabolism. Nature Genetics 1995;11:170-6.

Sauer B, Westphal H. Modulation of gene activity in the mouse mediated by Cre, a site-specific DNA recombinase. In: Lonai P,ed. Mammalian Development. Amsterdam: Harwood Academic Publ 1996;373-82.

Sheng HZ, Zhadanov AB, Mosinger B, Fujii T, Bertuzzi S, Grinberg A, Lee EJ, Huang SP, Mahon KA, Westphal H. Specification of pituitary cell lineages by the LIM-homeobox gene Lhx3. Science 1996;272:1004-7.

Wolfe AM, Wray S, Westphal H, Radovick S. Cell-specific expression of the human gonadotropin-releasing hormone gene in transgenic animals. J Biol Chem 1996;271:20018-23.

Project Numbers - Annual Report 1996

LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT (LMGD)

Z01 HD 00071-24	Gene and Transgene Regulation in the Developing Mouse Heiner Westphal, M.D.
Z01 HD 01801-06 **TERMINATED**	The Molecular Analysis of Murine Development Kathy Mahon, Ph.D.
ZO1 HD 01803-03	Genetic Analysis of Thymocyte Development Paul Love, M.D.
Z01 HD 01804-01	Genomic Imprinting on Distal Mouse Chromosome 7 Karl Pfeifer, Ph.D.

Z01 HD 01805-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00071-24-LMGD

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene and Transgene Regulation in the Developing Mouse

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: H. Westphal, M.D., Chief, LMGD, NICHD
Others: A. Agulnick, Ph.D., IRTA
S. Bertuzzi, Ph.D., VF
O. Birk, M.D., Ph.D., MSF
C. Chiang, Ph.D., GWF
S.P. Huang, Chemist
Y. Litingtung, Ph.D., IRTA
J. Pichel, Ph.D., VF
D. Porter, M.D., Ph.D., SSF
L. Shen, Ph.D., NRC1
H. Sheng, Ph.D., SSF
T. Yamashita, M.D., Ph.D., GW
Y. Yarden, Ph.D., Courtesy

COOPERATING UNITS *(if any)*

LMG, NICHD (I. Dawid); University of Colorado, Denver (B.J. Hoffer); University of Helsinki, Finland (M. Saarma, H. Sariola)

LAB/BRANCH

Laboratory of Mammalian Genes and Development

SECTION

Section on Transgene Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-2790

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
13	12	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Murine LIM-homeobox (Lhx) genes. This family of developmental transcription factors regulates important steps of organogenesis. Lhx2 and Lhx7, a pair of closely related genes, are essential for pattern formation, particularly in the developing central nervous system (CNS). Knockout analyses of these two genes are in progress to address their combinatorial functions in the CNS and elsewhere in the developing embryo. Lhx3 and Lhx4, another closely related pair of genes, is responsible for the formation of the adenohypophysis, and also plays an essential role in motoneuron innervation along the spinal chord, a function currently being assessed via double loss-of-function analysis.

LIM-binding proteins. We have isolated Ldb1, a novel factor that binds LIM motifs present in LIM-homeodomain and LMO proteins. In collaboration with the laboratory of Dr. Dawid , we have shown that the murine Ldb1 and its frog counterpart XLdb1 synergize with the LIM-homeobox gene 1 to induce early determinators of development and to form secondary axes when injected in frog embryos.

Glial cell line-derived neurotrophic factor (GDNF) was isolated as a neurotrophic factor for midbrain dopaminergic neurons. In view of its neurotrophic activity on a wide range of neuronal populations in vitro and in vivo, GDNF is being considered as a potential therapeutic agent for neuronal disorders. During mammalian development, GDNF is expressed not only in the nervous system, but also very prominently in the metanephric kidney and in the gastrointestinal tract, suggesting possible functions during organogenesis. We have investigated the role of GDNF during development by generating a null mutation in the murine GDNF locus. Together with the laboratories of Drs. Hoffer, Saarma and Sariola we were able to demonstrate that mutant mice show kidney agenesis or dysgenesis and defective enteric innervation. GDNF induces ureter bud formation and branching during metanephros development. In addition, the factor is essential for proper innervation of the gastrointestinal tract.

Project Description.

Objectives:

The goal common to all of our experiments is to learn details of mammalian gene regulation and control. Research topics addressed below include the process of oncogenesis and tissue differentiation, and the molecular genetics of embryonic development.

Methods Employed:

Characterization of DNA, RNA and proteins, gene cloning in prokaryotic vectors, microinjection of mouse zygotes, gene transfer into embryonic stem cells, transfer of embryonic stem cells into mouse blastocysts, transfer of zygotes and of blastocysts into foster mothers, zygote cryopreservation, in situ gene expression analysis of tissue sections.

Main Findings and Proposed Course:

Analysis of LIM/homeobox gene function. Homeodomain proteins have become well known for their function in pattern formation and cell type specification during metazoan development. The LIM/homeodomain proteins, discovered a few years ago, are characterized by LIM, a cysteine-rich motif that is present in two copies at the N-terminus of the peptide, in close vicinity of the homeodomain. We have isolated murine homologs of several members of the LIM/homeobox gene family, and have continued to characterize their function in mammalian development.

Lhx2, Lhx7 and Forebrain Development. As described in last year's report, our analysis of Lhx2 knockout mutant embryos has shown that Lhx2 function is necessary for eye, forebrain, and erythrocyte development. The forebrain developmental defect is especially interesting and requires further in situ analysis, employing multiple forebrain probes. The distinction between hypoplasia in the neocortex and aplasia of the archicortex may represent a functional demonstration of the prosomeric model of forebrain development, thereby reflecting a prosomere-specific difference among genes that interact with Lhx2 or that can substitute for its action. A yet to be characterized twin gene of Lhx2 might provide partial functional redundancy for Lhx2 in the neocortex, but not in the anlage of the hippocampus. The postulate of a twin gene was particularly attractive to us because the $Lhx2^{-/-}$ animals have no apparent abnormalities in other developmental fields, such as the limbs, where Lhx2 is highly expressed. Our search for genes with close sequence homology to Lhx2 has led to the cloning of a new gene that we have termed Lhx7. Like Lhx2, Lhx7 is expressed in the developing limbs, consistent with the idea of partial functional redundancy among twin genes. However, the forebrain expression pattern of Lhx7 is unique. We were excited to observe that neocortex expression of Lhx7 appears to be restricted to Cajal-Retzius cells. In order to be able to elucidate the individual and joint roles of Lhx2 and Lhx7 in neocortex development, we are currently generating a loss-of-function mutation in Lhx7.

Lhx3, Lhx4 and the development of the adenohypophysis. The progressive differentiation of distinct pituitary-specific cell lineages from a common primordium involves a series of developmental decisions and inductive interactions. Pituitary organ commitment occurs during the formation of Rathke's pouch when the ectoderm cells of the pouch come in contact with neuroectoderm of the ventral hypothalamus. Inductive interactions between these tissues are required for their proper determination and differentiation. Once committed to the pituitary fate, the epithelium lining Rathke's pouch gives rise to the adenohypophysis with its pituitary-specific cell lineages that secrete peptide hormones critical for homeostasis, growth, and reproduction. Our previous experiments had shown that Lhx3 is expressed in the adenohypophysis throughout development and in the adult. We are now able to state that Lhx3 is essential for the formation of this tissue. In mice homozygous for a deletion that removes the two LIM motifs and part of the homeobox of Lhx3, Rathke's pouch formed but failed to grow and differentiate; such mice lacked the entire adenohypophysis, i.e., the anterior and intermediate lobes of the pituitary. Pit-1, a transcription factor important for lineage development, was not activated, and the determination of all but one of the pituitary cell lineages was affected. Only the corticotrophs persisted in the non-proliferating pituitary primordium of the Lhx$^{-/-}$ mutants, suggesting that a distinct, Lhx3-independent ontogenetic pathway exists for the initial specification of this lineage.

Although these findings identify Lhx3 as a gene of pivotal importance for pituitary lineage determination, the fact that Rathke's pouch forms in the Lhx3$^{-/-}$ mutants suggested to us that a second gene may participate in the development of the adenohypophysis. The very close sequence homology and similarity of expression patterns between Lhx3 and Lhx4 prompted us to examine a possible role of Lhx4 in this context. We obtained the Lhx4 null mutant mouse from Dr. Potter's laboratory and observed a previously unnoticed reduction in the size of the adenohypophysis. Rathke's pouch forms in the Lhx4 mutant mice and all five anterior pituitary lineages are specified. However, the cell count is greatly reduced, indicating that Lhx4, like Lhx3, is indeed involved in the control of this organ. The fact that Rathke's pouch forms in both the Lhx3 and the Lhx4 null mutant suggests partial redundance in function at early stages of development of the adenohypophysis. Are the anlagen formed in the absence of both genes? To find out, we generated Lhx3/4$^{-/-}$ compound homozygotes. No pouch is observed in these embryos, an important fact confirming that both genes are required for the development of the adenohypophysis. Both genes are also prominently expressed in ventral fields of the developing spinal chord. Our future experiments will assess to which extend these genes contribute to motoneuron identity and axon projection.

LIM binding proteins and the mechanism of Lhx protein function. Our analysis of Lhx gene expression patterns and null mutant phenotypes is beginning to yield information concerning the role that individual members of this gene family play in mammalian development. This has encouraged us to start parallel efforts aimed at gaining insight into the mechanism of action of Lhx proteins. Toward this goal, we performed a protein-protein interaction screen with the intent of isolating mouse embryo derived cDNAs encoding LIM domain binding factors. Our preliminary findings are as follows. A truncated mouse Lhx1 peptide consisting of both LIM domains was partially purified and labeled with ^{32}P. This protein probe was used to screen a mouse E11 embryonic cDNA library known to express Lhx1. Five cDNA clones were isolated and partially sequenced, and four of these were found to be derived from the same gene. The clone with the most 5' sequence contained an open reading frame capable of encoding a 375 amino acid protein of predicted molecular weight 43 kD. This protein was designated Ldb1, for LIM-domain binding protein 1. Possible nuclear localization signals but no other known motifs were found in the sequence. Nuclear localization of Ldb1 has been shown in tissue culture and in injected Xenopus embryos. The fifth cDNA clone encoded a protein which is closely related to Ldb1, was named Ldb2 and will be analyzed later. A search of the databases revealed homology of Ldb1 and Ldb2 only to several human expressed-sequence tags of unknown function. To determine the binding specificity of Ldb1, Far-Western binding assays were performed. Full length LIM proteins were used to probe Ldb1 immobilized on filters. Mutation of a conserved cysteine residue in either LIM domain of Lhx1 abolished binding to Ldb1, indicating that the Lhx1-Ldb1 interaction is highly specific and requires two intact LIM domains. Specificity for binding to the various classes of LIM proteins was also examined. Ldb1 only binds to a related subgroup of LIM proteins, consisting of the LIM-HD and the LMO (Rhombotin/Ttg) proteins. An extensive NH2- and COOH-terminal deletion analysis of Ldb1 was performed to arrive at the preliminary conclusion that the C-terminal 150 amino acids specify much of the LIM binding activity.

Several experiments in Xenopus and in yeast have been started in collaboration with the laboratory of Dr. Igor Dawid (see also Z01 HD 01002-14 LMG). Using fusion proteins in a two hybrid system in yeast, we showed that Ldb1 can bind to the Lhx1 ortholog Xlim-1 in vivo, but not to a LIM domain mutant form of Xlim-1 indicating specific binding. When injected into Xenopus embryos, Ldb1 synergizes with Xlim-1 in forming partial secondary axes and in activating the genes encoding goosecoid (gsc), chordin, NCAM and XCG7, thereby demonstrating a functional interaction between the two proteins. Our observation that Ldb1 and Xlim-1 both localize to the nucleus is consistent with the idea that Ldb1 binds to Xlim-1 to mediate the observed synergistic activity. Given these results and the in vitro binding specificity of Ldb1, it is likely that Ldb1 plays an important role in the regulation of LIM-HD and LMO proteins.

In the forthcoming year, we will generate a knockout mutant of Ldb1 in order to gain insight into in vivo functions of this gene. At the same time, the biochemistry of protein complexes that involve LDB peptides will be analyzed in detail.

GDNF and the development of the kidney and the enteric nervous system. To examine developmental controls exerted by this glia cell line-derived neurotropic factor, we generated mutant mice carrying a GDNF allele that lacks the entire coding sequence. $GDNF^{+/-}$ were viable and fertile whereas $GDNF^{-/-}$ individuals, although born with the expected Mendelian frequency, died within 12-24 h after birth. $GDNF^{-/-}$ mutant mice displayed either renal agenesis or severe renal dysgenesis. Surprisingly, heterozygous $GDNF^{+/-}$ animals also showed renal abnormalities indicative of defective segmentation during development. Histological analysis of $GDNF^{-/-}$ embryos revealed that metanephric development is halted even though a morphologically distinct metanephric mesenchyme is present. No ureteric bud was found in most of the embryos. Where present, it had penetrated deeply into the mesenchyme, but the number of collecting ducts, nephric condensates and secretory nephrons were strongly reduced. Both types of defects, although less pronounced, were also seen in some of the $GDNF^{+/-}$ embryos. Analysis of the branching pattern in ex vivo cultured urogenital blocks dissected from E11.5 embryos revealed a significant reduction in the growing arborization of the renal collecting system of heterozygotes, and a delayed or total absence of ureteric branching in the $GDNF^{-/-}$ mutants. To examine whether GDNF was able to rescue the branching defect in mutant kidneys, we incubated explanted urogenital tissue in the presence of recombinant GDNF embedded in agarose beads. In vitro branching of the $GDNF^{-/-}$ ureters was increased significantly. Taken together, these results show that GDNF is a metanephric mesenchyme-derived factor, acting in a dose-dependent manner on the growth or arborization of the ureteric bud during early stages of kidney morphogenesis.

The second abnormality found in $GDNF^{-/-}$ mutant newborns was the presence of milk in the esophagus and reduced progression of food into the gastrointestinal tract, suggesting a defect in peristalsis. This raised the possibility that neural crest-derived enteric innervation might be affected by GDNF. Histological analysis of E18 embryos revealed that the neurons of the myenteric plexus, readily apparent in normal embryos, were absent from the intestine of $GDNF^{-/-}$ newborns. Whether GDNF is acting directly on multipotent neural crest-derived stem cells of the enteric neurons, or only on their survival after differentiation, remains to be defined.

Based on the reported neurotrophic action of GDNF, notably in dopaminergic and cholinergic neuron populations, we examined several brain areas in the GDNF mutants. Our preliminary analysis failed to reveal overt differences in either distribution, density, or tyrosine hydroxylase staining properties between substantia nigra dopamine neurons in newborns $GDNF^{-/-}$ mutant mice and the $GDNF^{+/+}$ controls. However, whether this holds for other brain stem nuclei as well, needs to be determined in more detail. Possibly, GDNF-like molecules can compensate for the lack of GDNF function in the mutant brain. Since our mutant mice die shortly after birth, we are unable to assess a possible role of GDNF in the maintenance of these neurons after birth.

Some human genetic disorders, notably Hirschsprung disease (HSCR) with megacolon formation are characterized by defects in gastrointestinal innervation. Mutations in the human RET locus have been demonstrated in some familial forms of HSCR. The loss of enteric neurons in $GDNF^{-/-}$ mice suggests the possibility that mutations in the GDNF gene may also cause HSCR. In addition, certain renal dysplasias that appear to result from a defect of ureteric branching might be linked to GDNF gene defects.

In summary, in vivo characterization of the GDNF mutant phenotype and ex vivo explant culture of mutant tissues indicate that GDNF has essential roles in ureteric budding and branching, and in the innervation of the gastrointestinal tract. It is entirely possible that the GDNF locus is implicated in human disorders in which target fields of this factor are affected.

Publications:

Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, José PA, Taylor SI, Westphal H. Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. Nature Genetics 1996;12:106-9.

Accili D, Fishburn CS, Drago J, Steiner H, Lachowicz JE, Park BH, Gauda EB, Lee EJ, Cool MH, Sibley DR, Gerfen CR, Westphal H, Fuchs S. A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. Proc Natl Acad Sci USA 1996;93:1945-9.

Albrecht FE, Drago J, Felder RA, Printz MP, Eisner GM, Sibley DR, Westphal H, Jose PA. Role of the D1A dopamine receptor in the pathogenesis of genetic hypertension. J Clin Invest 1996;97:2283-8.

Bertuzzi S, Sheng HZ, Copeland NG, Gilbert DJ, Jenkins NA, Taira M, Dawid IB, Westphal H. Molecular cloning, structure and chromosomal localization of the mouse LIM/homeobox gene Lhx5. Genomics 1996;36:234-9.

Chiang C, Litingtung Y, Lee EJ, Young K, Corden JL, Westphal H, Beachy P. Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function. Nature, in press.

Drago J, Gerfen CR, Westphal H, Steiner H. Differential regulation of substance P and immediate-early gene expression by cocaine in forebrain of D1 dopamine receptor-deficient mice. Neuroscience, in press.

Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee EJ, Alt FW, Westphal H. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci USA 1996;93:5860-5.

Lei KJ, Chen H, Pan CJ, Ward JM, Mosinger B, Lee EJ, Westphal H, Mansfield BC, Chou JY. Glucose-6-phosphatase substrate transport in the glycogen storage disease type-1a mouse. Nature Genetics 1996;13:203-9.

Levine MS, Altemus KL, Cepeda C, Cromwell HC, Crawford C, Ariano MA, Drago J, Sibley DR, Westphal H. Modulatory actions of dopamine on N-methyl-d-aspartate receptor-mediated responses are altered in D1A deficient mutant mice. J Neurosci 1996;16:5870-82.

Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H. GDNF is required for kidney development and enteric innervation. Cold Spring Harbor Symp Quant Biol 61, in press.

Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H. Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 1996;382:73-6.

Rankin T, Familiari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J. Mice homozygous for an insertional mutation in the Zp3 sperm receptor gene lack a zona pellucida and are infertile. Development 1996;122:2903-10.

Sango K, Yamanaka S, Hoffmann A, Okuda Y, Grinberg A, Westphal H, McDonald MP, Crawley JN, Sandhoff K, Suzuki K, Proia RL. Mouse models of Tay-Sachs and Sandhoff Diseases differ in neurologic phenotype, central nervous system pathology and ganglioside metabolism. Nature Genetics 1995;11:170-6.

Sauer B, Westphal H. Modulation of gene activity in the mouse mediated by Cre, a site-specific DNA recombinase. In: Lonai P,ed. Mammalian Development. Amsterdam: Harwood Academic Publ 1996;373-82.

Sheng HZ, Zhadanov AB, Mosinger B, Fujii T, Bertuzzi S, Grinberg A, Lee EJ, Huang SP, Mahon KA, Westphal H. Specification of pituitary cell lineages by the LIM-homeobox gene Lhx3. Science 1996;272:1004-7.

Wolfe AM, Wray S, Westphal H, Radovick S. Cell-specific expression of the human gonadotropin-releasing hormone gene in transgenic animals. J Biol Chem 1996;271:20018-23.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01801-06-LMGD

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Molecular Analysis of Murine Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

K.A. Mahon, Ph.D., Expert
D.E. Feltner, M.D., Guest Worker
T. Hatta, M.D., Guest Worker
E. Hermesz, Visiting Associate
L. Kos, Ph.D, Visiting Fellow
M.A. O'Reilly, Ph.D., NRC Fellow

COOPERATING UNITS *(if any)*

CBER, FDA (M. Jamrich), LP, NCI (S. Mackem), LMG, NICHD (T. Sargent), U. of Michigan (S.Camper)

LAB/BRANCH

Laboratory of Mammalian Genes and Development

SECTION

Unit on Developmental Gene Regulation

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project has been terminated.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01803-03 LMGD

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic Analysis of Thymocyte Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Paul E. Love	Section Head	LMGD: NICHD
Others:	Connie L. Sommers	IRTA Fellow	LMGD: NICHD
	Hala Azzam	IRTA Fellow	LMGD: NICHD
	Kun Huang	Biologist	LMGD: NICHD
	Howard Shen	Pre-IRTA	LMGD: NICHD
	Kin Lui	Pre-IRTA	LMGD: NICHD
	Chris Muntzel	HHMI Fellow	LMGD: NICHD

COOPERATING UNITS *(if any)*

CBER, FDA (E.W. Shores) STR, LMGD, NICHD (H. Westphal, A. Grinberg, E.J. Lee)
LMAI, NIAID (V. Flammand, J.-P. Kinet) LMI, NHLBI (X. Cao, W.J. Leonard)

LAB/BRANCH

Laboratory of Mammalian Genes and Development

SECTION

Unit on Cellular and Developmental Biology

INSTITUTE AND LOCATION

NICHD, NIH 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.25	3.0	3.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Research is directed at investigating the cellular and genetic events that control normal T cell development. Transgenic and gene-targeting methods are used to analyze the function of known genes and various techniques (e.g.,RT-PCR, gene cloning) are being employed to identify novel genes that participate in thymocyte development. Current studies are focused on: (I) Examining the role of T cell antigen receptor (TCR) signal transduction in thymocyte maturation. In mature T cells, the TCR transduces signals important for T cell activation and cell mediated immunity. In immature T cells the TCR is required for thymocyte development and for thymic (positive and negative) selection. The TCR is composed of multiple signal transducing subunits (the CD3 chains and one or more members of the ζ-family of proteins; ζ, η and Fcγ) that couple the TCR to intracellular signal transduction pathways via conserved functional sequences (*I*mmunoreceptor *T*yrosine based *A*ctivation *M*otifs; ITAMs). To address whether these signal transducing subunits perform distinct or analogous functions in development we have: a) examined their role in T cell ontogeny by generating ζ/η-deficient andζ/η/Fcγ deficient mice by gene targeting, and then genetically reconstituting these mice with transgenes encoding the individual ζ-family proteins, and b) examined the function of the 3 individual ζ-chain ITAMs in thymocyte development and selection by transgene reconstitution of ζ-deficient mice. These studies revealed that expression of ζ chain (or a related ζ-family protein) is required for TCR surface expression but that ζ chain signals are not specifically required for T cell development. Thus the CD3 subunit ITAMs can transduce all of the signals necessary for thymocyte maturation. However, a direct relationship was observed between the number of TCR-ζ ITAMs and the efficiency of both positive and negative thymocyte selection suggesing that the multiple TCR ITAMs function primarily in signal amplification. These findings demonstrate a previously unrealized role for multiple TCR ITAMs in thymocyte selection and identify a function for signal amplification in selection of the T cell repertoire. (II) The role of other signal transducing proteins in T cell development is being examined by the generation of transgenic/knockout mice. These include the CD3ε subunit of the TCR and CD5 a distinct surface receptor that also contains an ITAM-like sequence. (III) Novel genes that have potential functions in thymocyte development or T cell activation are being identified by RT-PCR. A new lymphoid-specific PTK, Txk has been cloned and biochemical and transgenic approaches are being employed to analyze its function. A similar approach has been used to identify other genes involved in early T cell development.

Project Description: Genetic analysis of T Cell Development

Objectives: To understand the cellular and molecular events that control T cell development and formation of the mature T cell repertoire.

Methods Employed: Transgenic and gene targeting methods are used to create overexpression,-dominant-negative, and loss-of-function mutants to investigate the role of specific genes in T cell ontogeny. Molecular and genetic techniques (including generation and screening of cDNA libraries, PCR and cloning) are also being used to identify novel genes that function in thymocyte development. Biochemical studies include: cell surface radioiodination / immunoprecipitation / western blotting / 1- and 2-dimensional SDS-PAGE, NEPHGE. Cell culture techniques include: fetal thymic organ culture and transfection of established cell lines. Flow cytometric analysis of thymocytes and peripheral T cells. Immunohistochemistry and *In situ* hybridization.

Background: T cells develop throughout the life of an individual. Progenitor cells originate in the fetal liver or adult bone marrow and then migrate to thymus where they mature and reach their full potential. The most immature thymocytes are designated double negative (DN) as they do not express the CD4 and CD8 coreceptors. Rearrangement of the T cell antigen receptor (TCR) β locus is initiated in DN thymocytes and triggers a seemingly irreversible lineage commitment leading to the formation of cells that express both CD4 and CD8 (double positive, DP). Rearrangement of TCRα occurs in DP thymocytes and these cells then express surface αβTCR. DP thymocytes then undergo positive and negative selection, a process that directly depends upon the specificity of their TCR and that leads to the survival of competent "self-educated" cells, deletion of autoreactive cells and death of nonresponsive cells. The ultimate product of the thymocyte maturational pathway is formation of mature functional cells that express CD4 or CD8 but not both (i.e., single positive, SP) and high levels of surface αβTCR.

Differentiation of precursor thymocytes into mature, functional T cells is controlled in part by signals delivered through the TCR. In particular, TCR mediated signals regulate positive and negative selection in the thymus, and are required for the differentiation of immature thymocytes into mature functional T cells. The TCR complex that is expressed on DP and SP thymocytes and most T cells is composed of six different subunits that function either in antigen recognition or signal transduction. The clonotypic TCRα/β chains are responsible for ligand specificity and are generated by productive rearrangement of germline gene segments, a process mediated by the products of the Rag-1 and Rag-2 genes . The α/β heterodimer lacks inherent signaling activity but associates noncovalently with multiple signal transducing subunits: the CD3-γ,-δ and -ε components, and a ζ chain homodimer. ζ belongs to a family of proteins (ζ , η and FcεR1γ) that are structurally and functionally related. The CD3 and ζ family proteins contain semi-conserved sequences within their cytoplasmic domains, termed *I*mmunoreceptor *T*yrosine-based *A*ctivation *M*otifs (ITAMs) that couple the TCR to intracellular signal transduction pathways by their ability to interact with protein tyrosine kinases. The CD3 and FcεR1γ chains each contain a single ITAM, η contains two ITAMs and ζ contains three ITAMs.

The rationale for multiple TCR signal transducing subunits has been enigmatic. In particular, it is unclear whether the ζ and CD3 molecules perform identical or distinct signaling functions, or if the relative contribution of these molecules varies with the developmental stage of the T cell. The multisubunit nature of the TCR suggests the potential for variability in the signaling response depending upon its configuration. Indeed, a precursor form of the TCR (the pre-TCR) is expressed on DN thymocytes. The pre-TCR, which is expressed prior to rearrangement of TCRα, consists of the β chain paired with a pre-Tα chain (pre-Tα) and the CD3 chains; ζ chain either does not associate with the pre-TCR or is only loosely associated with this complex. It has also been shown that the TCRs expressed on a subpopulation of mature T cells (γδ T cells) contain both ζ and FcεR1γ chains raising the possibility that inclusion of different ζ-family molecules as part of the TCR complex could result in the transduction of distinctive signals following TCR engagement.

We have begun to investigate the role of the TCR signals in T cell development and T cell function by analyzing the *in vivo* function of the various TCR signal transducing subunits. Our approach has been to use transgenic and gene targeting methods to create overexpression, dominant-negative, and loss-of-function mutants.

Major Findings:

1. Role of TCR signaling molecules in T cell development (in collaboration with EW Shores, FDA.)

a. Overexpression of ζ-family dimers in transgenic mice. Transgenic mice that overexpress each of the ζ-family proteins (ζ , η or FcεR1γ) were generated in our facility. Analysis of ζ transgenic (ζTg) mice yielded the unexpected finding that thymocyte development was arrested at the early DN→DP precursor stage. Further investigation revealed that overexpression of ζ-chain caused premature termination of Rag-1 and Rag-2 expression in early thymocytes, preventing productive rearrangement of the TCRα and TCRβ genes. Interestingly, overexpression of truncated ζ chains or other ζ-family proteins (η, FcεRIγ) in early thymocytes did not result in downregulation of Rag expression, indicating that this effect was specific to ζ. Down-regulation of Rag-1 and Rag-2 in ζTg mice appears to be the result of ζ-mediated signaling; coinciding with the down-regulation of Rag transcripts, DN thymocytes from ζ transgenic mice express inappropriately high levels of the CD5, CD2 and CD69, antigens whose expression is known to increase upon T cell activation. Interestingly, the effects of ζ-chain overexpression (i.e., thymocyte activation and downregulation of Rag-1 and Rag-2) are similar to those normally observed in $\alpha\beta TCR^+$ DP thymocytes as they undergo thymic selection and then mature into SP thymocytes. Signals transduced by the αβTCRs on DP thymocytes regulate expression of Rag-1 and Rag-2 as direct engagement of αβTCR complexes on DP thymocytes results in down-regulation of Rag-1 and Rag-2. The TCR mediated termination of Rag expression in DP thymocytes is thought to be a critical step in late thymocyte development as it effectively results in allelic exclusion of TCRα, thus fixing the specificity of the αβTCR. However, the early developmental arrest in ζTg mice was not due to failure to rearrange TCRα and TCRβ genesas it was not alleviated by the introduction of functionally rearranged TCRα and TCRβ transgenes. Therefore, the thymocyte developmental arrest in these mice is the result of stage-inappropriate signaling which blocks further thymocyte maturation

Immature DN thymocytes express a precursor form of the TCR pre-TCR complex (pre-TCR) which consists of TCRβ, a pre-Tα chain and the CD3 chains. The pre-TCR either does not associate with ζ or only weakly associates with ζ. If ζ-chain were part of the pre-TCR complex (as we predict may occur in transgenic mice overexpressing ζ), signals mediated by this complex could prematurely down-regulate Rag expression preventing rearrangement of TCRα. These results suggest that a ζ-less pre-TCR complex may be necessary for the transduction of signals which support early thymocyte development, but which do not prematurely terminate Rag expression. Signaling through the pre-TCR controls differentiation of DN thymocytes into DP thymocytes and also controls allelic exclusion of TCRβ. Therefore, the configurations of the pre-TCR and the αβTCR appear to be particularly well adapted to transduce signals that control allelic exclusion of the TCRβ and TCRα genes, respectively. Our results indicate that inclusion of ζ chain as a signaling component of these surface complexes may be developmentally regulated so that the appropriate signals are delivered to thymocytes at specific stages of development.

Analysis of transgenic mice overexpressing FcεR1γ chain revealed a phenotype quite different than that of ζ overexpressing mice. Interestingly, these mice exhibit delayed development of both αβ T cells and TCR^- Natural Killer (NK) cells. However, unlike ζTg mice, downregulation of Rag-1 and Rag-2 did not occur in γTg mice. DN thymocytes from FcεR1γ-transgenic mice express unusually high levels of

CD16 (FcγRIII) a receptor that is normally expressed on NK cells in association with FcεR1γ. T cells and NK cells are thought to be derived from a common (DN, TCR^- $CD16^+$) precursor in the thymus. Thus, our results suggest that signals mediated by FcεR1γ may control very early steps in thymocyte development that preceed expression of the TCR complex and thus indicate that individual ζ-family proteins may play specialized roles in T cell development.

b. Generation of $\zeta^{-/-}$ and $\zeta^{-/-}$;Tg mice. Examination of mice in which the gene encoding ζ-chain was inactivated by homologous recombination ($\zeta^{-/-}$) revealed the importance of this protein in normal T cell development. Thymi from $\zeta^{-/-}$ mice are markedly reduced in size and cellularity and the phenotype of thymocytes reveals that in the absence of ζ-chain, T cell development is inhibited. In addition, thymocytes and T cells from $\zeta^{-/-}$ mice express barely detectable levels of TCR on their surface. SP thymocytes are almost undetectable in $\zeta^{-/-}$ mice, however, a small number of these cells can be identified. Yet, despite the almost complete lack of SP thymocytes in the thymus, the periphery of these animals contain low, but readily detectable number of SP T cells that seem to accumulate with age. The apparently normal ratio of $CD4^+$ to $CD8^+$ T cells supports the idea that these cells are thymically derived. The forces driving the development of these SP cells remain unclear since they express extremely low levels of surface TCR. One possibility is that SP cells develop through a TCR independent pathway, perhaps involving signals through molecules such as Thy1, Ly6, CD2 or CD5. However, it may not be necessary to invoke alternative signaling pathways as we were able to detect low levels of CD3ε and TCRβ on the surface of T cells from $\zeta^{-/-}$ mice by flow cytometry. Moreover, we have found that DP thymocytes from $\zeta^{-/-}$ mice express low levels of signaling-competent TCR complexes. Engagement of the TCRs on DP thymocytes from $\zeta^{-/-}$ mice with antibodies directed against the TCRβ chain leads to signaling events associated with positive selection. Therefore, we favor the idea that the generation of SP thymocytes is a consequence of TCR mediated signals delivered through a "nonclassic" TCR composed of α ,β and CD3 chains, but lacking ζ.

These findings were quite striking and unexpected as they suggested that a ζ-less TCR complex could deliver signals sufficient for the positive selection of at least some thymocytes. The relative inefficiency of this process (as reflected by the number of SP cells generated) could be reflective of the extremely low level of TCR expression in the absence of ζ. Interestingly, despite the ability of ζ-less TCRs to promote T cell development, peripheral T cells from $\zeta^{-/-}$ mice fail to proliferate in response to mitogens or TCR engagement. These findings could indicate an anergic state of these cells, as might be induced by TCR engagement in the absence of costimulatory requirements (e.g. IL-2). Another interpretation is that signals required for positive selection may be quantitatively or qualitatively different than those signals required for activation of peripheral cells.

Because ζ is required for efficient surface expression of the αβTCR complex, it was impossible to discern whether the developmental defects in ζ-deficient mice were due specifically to the lack of ζ-mediated signaling or to the failure to target other TCR subunits to the cell surface. Thus, although the phenotype of ζ-deficient mice revealed that ζ chain plays an important role in thymocyte development, the relative significance of ζ-mediated signals, individual ζ-chain signaling motifs (ITAMs), or CD3 subunit-mediated signals could not be adequately addressed in $\zeta^{-/-}$ mice. To examine this question we reconstituted $\zeta^{-/-}$ mice with ζ chains that either partially or completely lacked sequences required for signal transduction. We generated a set of transgenes that encoded either full length ζ chain or genetically engineered variants of ζ that lacked one or more ITAMs. The transgenes were placed under the control of identical regulatory sequences, and since they all retained the extracellular and transmembrane ζ chain domains, the encoded proteins were able to dimerize, assemble with other TCR components and fully restore surface TCR expression, thus enabling a direct analysis of the role of ζ-chain signals in T cell development.

Remarkably, all of the transgene encoded ζ-chain variants, most notably the variant which lacked all three ITAMs, alleviated the block in thymocyte development. Numbers of DP and SP thymocytes were markedly increased in these mice relative to $\zeta^{-/-}$ mice. Restoration of normal or near-normal numbers of SP thymocytes and peripheral T cells in all of the reconstituted lines indicated that even TCR complexes that contained signaling-deficient ζ chains (0 ITAMs) were capable of supporting positive selection of thymocytes. Moreover, these TCR complexes were competent to trigger cellular responses directly associated with positive selection, such as downregulation of Rag-1 and Rag-2 mRNA. Similar results were obtained using ζ-deficient mice reconstituted with either the η or FcεR1γ chain. Thus, these experiments firmly established that the ζ-chain ITAMs are not specifically required for thymocyte development. In addition, the ability of the signaling-deficient ζ chain to rescue thymocyte development in $\zeta^{-/-}$ mice demonstrated that, providing TCR surface expression was restored to normal levels, signals transduced by the CD3 subunits are sufficient to mediate thymocyte maturation and selection.

c. Role of the TCR ITAMs in thymocyte positive and negative selection. Although the genetic reconstitution experiments did not identify a qualitative difference between individual ζ or CD3 subunit ITAMs, they did reveal more subtle quantitative effects. At similar levels, TCR complexes containing full length ζ-chains were more efficient at reconstituting T cell development (as judged by numbers of SP thymocytes and peripheral T cells) than complexes containing ζ-chains that lacked ITAMs. Thus, these data indicated that the multiple ζ-chain ITAMs may function primarily to amplify TCR signals. Signal amplification might be important for establishing the T cell activation threshold, but perhaps more importantly, for regulating thymocyte positive and negative selection. During development, the fate of thymocytes is ultimately dictated by their clonotypic TCR and the signals it transduces. However, it has been difficult to understand how signals delivered through the same TCR complex can differentially lead either to positive or negative selection. While a number of models have been proposed to explain these two different outcomes of TCR engagement, models based on the quantity or strength of the TCR signal seem most compatible with the results obtained with transgene-reconstituted $\zeta^{-/-}$ mice. Several factors that appear to quantitatively influence TCR signaling include 1) the nature of the ligand and how well it "fits" with the TCR, 2) the quantity of ligand thus influencing the number of TCRs engaged, and 3) the level of TCR or coreceptor (CD4 or CD8) surface expression. Whereas these models have primarily focussed on the nature of the TCR-ligand interaction, their prediction is that decreased or enhancing signaling can influence whether signals delivered through the TCR will result in positive or negative selection.

Recent data from our laboratory provide strong support for the quantitative signaling model. We have bred into $\zeta^{-/-}$; Tg -reconstituted mice TCRαβ transgenes. The TCRαβ transgenes inhibit rearrangement of endogenous TCRα and TCRβ genes, thereby producing a nearly uniform population of thymocytes with identical TCRs. The use of the TCRαβ transgenic system is extremely powerful for assessing the effects of other factors on positive and negative selection as all thymocytes exhibit the same TCR specificity. Because the transgenic αβTCR uses in this study recognizes an antigen (H-Y) expressed only in males, virtually all thymocytes are positively selected in female mice but are negatively selected in male mice. The results of these experiments revealed a direct relationship between the number of ζ-chain ITAMs in the TCR complex and the efficiency of both positive and negative thymocyte selection. In addition, they indicated that the multiple ζ-ITAMs function primarily to amplify signals generated by the TCR during thymocyte selection. In this respect, our findings are consistent with *in vitro* results obtained from mature T cell clones which failed to identify a specific function for individual ζ ITAMs but found that triplication of a single motif resulted in a quantitative enhancement of signaling. These results also directly address a major question in developmental immunology. It has remained unclear how relatively small differences in the affinity or avidity of TCR self-ligand interactions can differentially lead either to positive or negative selection. Our data suggest that the ability of the TCR to elicit such developmentally distinct cell fate decisions stems from its unique structure. Multiple ITAMs may serve to greatly magnify TCR signals and thereby translate relatively small differences in avidity into large signaling differences. Thus, the unique configuration of the TCR, with its capacity for signal amplification, plays a pivitol role in determining the ultimate fate of developing thymocytes.

d. Generation and analysis of ζηγ-deficient mice. (in collaboration with EW Shores, FDA and

JV Ravetch, Rockefeller University). We have generated mice that lack expression of all ζ-family proteins by mating our $\zeta/\eta^{-/-}$ mice to $Fc\varepsilon R1\gamma^{-/-}$ ($\gamma^{-/-}$) mice (provided by Dr. J. Ravetch). These $\zeta/\eta^{-/-}$ x $\gamma^{-/-}$mice have been especially useful in examining γδT cell development. In $\zeta/\eta^{-/-}$ mice, $\gamma\delta TCR^{+}$ intestinal intraepithelial lymphocytes (iIELs) and dendritic epidermal T cells (DETCs) express surface TCR at relatively high levels, whereas all peripheral and intestinal αβ T cells are $TCR^{lo/-}$. Biochemical studies indicate that in wild-type mice these $\gamma\delta TCR^{+}$ T cells express TCRs that are composed of both γ and ζ chains, whereas the TCRs expressed on $\alpha\beta TCR^{+}$ T cells are composed of only ζ chains. Thus the continued expression of γ chain in γδT cells from $\zeta/\eta^{-/-}$ mice enables expression of TCRs associated with γγ dimers. Consistent with this observation, we have found that in double knockout ($\zeta/\eta^{-/-}$ x $\gamma^{-/-}$) mice iIELs and DETCs are present but do not express detectable surface (αβ or γδ) TCRs. The reason for the different TCR signaling subunit structures expressed on $\alpha\beta TCR^{+}$ and $\gamma\delta TCR^{+}$ T cells remains unclear. γδ T cells are involved primarily in mucosal immunity and at least some of these cells are thought to arise through a thymic-independent developmental pathway. Future experiments will specifically address the signaling function of the TCRs on γδT cells by generating mice whose γδIELs express only ζ-chain ($\gamma^{-/-}$ mice) or γ-chain ($\zeta^{-/-}$ mice).

2. Role of CD5 in T cell Development..

CD5 is a 67 kDa cell surface protein expressed on thymocytes and mature T cells. Several recent studies have suggested a role for CD5 in T cell activation and thymocyte selection. Like ζ, CD5 contains a di-tyrosine motif (ITAM-like) within its intracellular domain. We have characterized the expression of CD5 during T cell development and found that the level of CD5 expression correlates with the developmental stage of thymocytes. Moreover, CD5 expresion is upregulated in response to TCR cross-linking. Interestingly, overexpression of a CD5 transgene does not affect thymocyte development in the B6 background. However, analysis of CD5 Tg x H-Y TCR Tg female mice revealed a profound effect of CD5 overexpression on positive selection. Increased expression of CD5 inhibited positive selection in H-Y females indicating that in contrast to ζ, CD5 signals are normally inhibitory. This observation is also supported by the work of another group which found increased positive selection in the absence of CD5 (i.e., in $CD5^{-/-}$ x H-Y Tg female mice). These investigators also found that $CD5^{-/-}$ thymocytes were hyperrresponsive to TCR stimulation. Collectively, these results suggest that the function of CD5 may be similar to two other inhibitory cell surface signaling molecules (eg., FcγRII and CD22) that contain di-tyrosine ITAM-like sequences termed Immuno-receptor-tyrosine-based-inhibition-motifs (ITIMs). FcγRII and CD22 are expressed on B cells and function to block signaling through the B cell receptor, presumably by binding an SH2-domain containing protein tyrosine phosphatase (PTP1C or SHP1). Thus, CD5 may perform a similar function in T cells. Future experiments will explore this possibility further.

3. Identification of novel genes involved in T cell development.

We recently initiated a search for novel genes that may be important for thymocyte maturation. Using a reverse-transcriptase PCR (RT-PCR)-based strategy that employs degenerate oligonucleotides specific for certain classes of proteins (including protein tyrosine kinases, protein tyrosine phosphatases and homeobox genes), cDNA from early fetal (day 13) or adult thymus is being screened.

a. Txk. We have identified a novel nonreceptor protein tyrosine kinase gene expressed in early thymocytes (*txk*). Txk contains SH2, SH3 and kinase catalytic domains and belongs to the Tec family of protein tyrosine kinases. Txk is expressed in thymocytes as early as fetal day 13.5 and its expression continues throughout development. *txk* transcripts are present in peripheral T cells and in mast cell lines but are not detectable in B cell, macrophage /monocyte cell lines or in non-hematopoietic fetal or adult tissues. In both thymocytes and T cells *txk* transcripts are downregulated after activation by TCR cross-linking but not by treatment with IL-2 which stimulates cell proliferation. Expression of Txk in COS cells confirms its cytoplasmic localization and kinase activity. Antipeptide antibodies have been made to Txk. Using these antibodies, we have immunoprecipitated Txk from thymocytes and T cells and can demonstrate intrinsic PTK activity. Transgenic mice that overexpress wild-type and dominant negative (kinase dead) forms of Txk have been generated to determine the possible role of this PTK in T cell development. Preliminary results indicate that Txk may be involved in regulating thymocyte positive selection.

b. PTPK1. A novel protein tyrosine phosphatase (PTP) was also identified by RT-PCR of fetal thymus cDNA. The sequence of this gene (PTPK1) predicts a cytoplasmic protein without SH2 or SH3 domains. The expression of PTPK1 is rather broad and includes bone marrow, liver, kidney thymus and brain.

Proposed course of projects.

1. Role of TCR signaling molecules in T cell development. We have recently generated a targeted mutation of the TCR-CD3ε gene in embryonic stem cells by homologous recombination and have also made transgenic mice that express either wild-type or mutant (ITAM-less) forms of the CD3ε chain to analyze the function of this TCR subunit in T cell development. These studies are viewed as complementing our ongoing effort to elucidate the role of TCR signals in T cell development and T cell function. We ultimately envision generating mice lacking both ε and ζ ($\varepsilon^{-/-}$ x $\zeta^{-/-}$). Transgene-reconstitution of these double knockout mice with full length ζ plus signaling deficient ε transgenes or signaling deficient ζ plus full length ε transgenes will enable us to specifically determine the function of the ζζ and CD3 (γεδε) TCR signal transduction modules, respectively. In addition to examining the role of these TCR signaling modules in thymocyte maturation and selection, we will begin to systematically examine the function of mature T cells from these mice. Mature T cell functions that will be analyzed include: T cell activation & proliferation, cytotoxic T cell (CTL) activity, cytokine production, and T-dependent B cell responses. Finally, it will be especially interesting to examine Thelper (Th1 and Th2) subsets and Th lineage commitment in these mice. The ability to elicit primarily Th1 or Th2 (which are differentiated by the type of cytokines that are elaborated) T cell responses correlates with resistance or susceptibility to several pathogens (eg., leishmania and HIV) and has been associated with certain autoimmune diseases. Understanding the signals that drive the immune response towards a predominantly Th1 or Th2 profile is therefore critical to elucidating the pathogenic mechanisms underlying these human diseases.

2. CD5. Further characterization of the role of CD5 in thymocyte development and selection will be directed in three areas: 1) The function of CD5 in positve selection will continue to be assessed by examining multiple αβTCR Tg x CD5 Tg lines. Preliminary data indicate that CD5 levels are different (varying from low to high) between different αβTCR Tg lines. Moreover, the effect of CD5 overexpression also varies (i.e., positive selection in strongly inhibited in αβTCR Tg mice whose thymocytes normally express low levels of CD5, but minimally affected in αβTCR Tg mice whose thymocytes normally express relatively high levels of CD5). These results suggest that the surface level of CD5 may be adjusted or fixed during development in accordance with the affinity of the TCR (i.e., thymocytes with lower affinity TCRs that are positively selected express low levels of CD5 and vice versa). 2) We are currently reconstituting $CD5^{-/-}$ mice with transgenes that express low or high levels of CD5 and with a transgene that lacks the putative functional (ITIM) domain of CD5. These mice will enable us to systematically examine the role of CD5 in thymocyte development and selection and will allow us to determine if the cytoplasmic ITIM domain of CD5 is important for its inhibitory function. 3) We are initiating biochemical experiments aimed at identifying cellular proteins that interact with CD5. These experiments

include co-immunoprecipitation with anti-CD5 following T cell activation and binding of extracts from activated thymocytes or T cells to GST-CD5 fusion proteins. Candidate binding proteins such as SH2-containing PTPs will be directly screened using antibodies to these proteins. Finally, if an association is detected in these experiments, it can be tested using the $CD5^{-/-}$; CD5 Tg and $CD5^{-/-}$; mutantCD5 Tg mice as the former but not the latter (which only express the tailless form of CD5) should also show a physical association.

3. *Txk* The restricted expression pattern of *txk* and its regulation by T cell activation make this gene an excellent candidate for involvement in signal transduction during T cell development. Ongoing characterization of *txk* will include work with stable transfectants obtained in established T cell lines (BW, 2B4 and Jurkat). These clones will be used to search for associated proteins (substrates) and to further characterize the regulation of Txk and its possible role in T cell activation. In addition, we are continuing to analyze transgenic mice overexpressing wild-type and dominant negative (kinase dead) forms of Txk to determine its possible role in thymocyte positive selection.

4. Genes controlling early thymocyte development. We have also utilized RT-PCR to identify potentially novel homeobox containing genes in the early fetal thymus. These experiments are especially attractive as they address potential candidates for early control of T cell lineage committment or thymus development and because they integrate well with similar ongoing experiments in the Section on Transgene Regulation. We designed primers for several subfamilies of homeobox genes, and have obtained PCR products in all cases. We are currently concentrating first on the *Antennapedia* subfamily of homeobox genes. Two novel homeobox sequences were obtained from fetal thymus. These genes are thus potential candidates for involvement in thymus/thymocyte development. We are currently screening fetal thymius cDNA libraries for the full length clones of these genes.

Publications:

Combadiere, B, Freedman, M, Chen, L, Shores, EW, Love, PE, Lenardo, MJ. Qualitataive and quantitative contributions of the T cell receptor ζ chain to mature T cell apoptosis. J Exp Med 1996;183:2109-17.

Flamand, V, Shores, EW, Tran, T, Huang, K, Lee, E, Grinberg, A, Kinet, J-P, Love, PE. Delayed maturation of $CD4^-$ $CD8^-$ $Fc\gamma RII/III^+$ T- and NK- cell precursors in FcεRIγ transgenic mice. J Exp Med, in press.

Huang, K, Sommers, CL, Grinberg, A, Kozak, CA, Love, PE. Cloning and characterization of PTPK1, a novel nonreceptor protein tyrosine phosphatase highly expressed in bone marrow. Oncogene, in press.

Khattri, R, Sperling, AI, Qian, D, Fitch, FW, Love, PE, Bluestone, JA. TCRγδ cells in CD3ζ-deficient mice contain FcεR1γ in the receptor complex but are specifically unresponsive to antigen. J Immunol, in press.

Shores, EW, Love, PE. Insights into T cell development and signal transduction provided by TCR-ζ chain deficient mice. Int Rev Immunol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01804-01 LMGD

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genomic Imprinting on Distal Mouse Chromosome 7

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Karl Pfeifer	Section Head	LMGD: NICHD
Others:	Madhulika Srivastava	Visiting Fellow	LMGD: NICHD
	Salil Goorha	Pre-IRTA Fellow	LMGD: NICHD
	Todd Gould	Pre-IRTA Fellow	LMGD: NICHD
	Elizabeth Ives	Pre-IRTA Fellow	LMGD: NICHD

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Mammalian Genes and Development

SECTION

Unit on Genomic Imprinting

INSTITUTE AND LOCATION

NICHD, NIH 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.3	1.1	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Normal mammalian development requires the contribution of haploid genomes from both parents, indicating that the two genomes are not functionally equivalent. This non-equivalence is the result of gamete-specific epigenetic modifications of a number of genes that lead to the unequal expression of the two parental alleles during development, a phenomenon known as imprinting. In humans, several developmental disorders are associated with disruption of the normal patterns of genomic imprinting. One specific example is Beckwith-Wiedemann Syndrome which is associated with disruption of imprinting at 11p15.5. Our group is investigating molecular mechanisms for gene regulation on mouse distal chromosome 7, a region syntenic with human 11p15.5, in order to understand how the cell distinguishes parental origins of its chromosomes and how this distinction is manifested in unequal expression of the two chromosomes. Mouse distal 7 contains at least 5 imprinted genes: *p57KIP2, Mash-2, Ins-2, Igf-2,* and *H19.* Our approach toward understanding regulation in this region takes three directions. First, we are investigating the molecular mechanisms for imprinting of the *H19* gene. We have introduced a 16 kb DNA fragment carrying the *Mus spretus H19* gene into heterologous locations in the genome and demonstrated that multiple copies are sufficient for paternal silencing and DNA methylation. Replacing the *H19* structural gene with a luciferase reporter gene results in loss of imprinting of the transgene. The removal of 701 base pairs at the 5' end of the structural gene results in a similar loss of parental-specific DNA methylation demonstrating that these sequences are required for both the full establishment and maintenance of the sperm-specific gametic mark. Expression of two additional transgenic constructs demonstrates that sequences in the *H19* structural gene further upstream are not absolutely required for imprinting. Current experiments are designed to distinguish between a role for the 5' sequences in cis or in trans for transgene imprinting. Previous work has demonstrated that *H19* is a regulatory locus, required for silencing of the neighboring insulin genes on the maternal chromosome. We have generated knockout mice that will distinguish between two possible models explaining this requirement for *H19:* competition between the promoters of the insulin genes and of *H19* versus the action of a methylation sensitive boundary element. Finally, we are generating a physical map and contig of the region between *p57KIP2* and *H19.* We will use this DNA to search for other imprinted genes in the region. In addition, we will generate transgenic mice to investigate the mechanisms for imprinting of *p57KIP2* in order to ascertain the generality of the mechanisms noted at the *H19* locus.

PHS 6040 (Rev. 5/92)

Project Description:

Objective: Genomic imprinting is a crucial aspect of normal mammalian development and represents an excellent model system for examining the role of chromosome structure and genome organization in gene expression. The goal of our unit is to understand the molecular mechanism for allele restricted expression using distal mouse chromosome 7 as our model system.

Introduction:

The *H19* gene lies in a cluster of at least five imprinted genes on mouse distal chromosome 7. The genes encoding *p57KIP2,* a cyclin-dependent kinase inhibitor, and *Mash-2,* a trophoblast-specific transcription factor, lie at the telomeric end of the cluster and are expressed only from the maternally inherited chromosome. The two growth factor genes, *Ins-2* (insulin-2) and *Igf-2* (insulin-like growth factor-2), lie in the middle of the cluster and are expressed from the paternal chromosome. *H19* resides at the centromeric end of the cluster and encodes a maternally expressed RNA. A mutation deleting the *H19* gene and 10 kb of upstream sequences results in loss of imprinting (i.e. biallelic expression of the two growth factor genes) indicating that *H19* acts as a regulatory locus. Disruption of imprinting at the syntenic region in humans,11p15.5, has been associated with Beckwith Wiedemann Syndrome and with Wilms tumor.

Major Findings:

1. Position independent expression and imprinting of H19 transgenes.

We have previously shown (with Shirley Tilghman at Princeton University) that a 15 kb DNA fragment carrying the *H19* structural gene and 4 kb of 5' flank and 8 kb of 3' flank is sufficient to direct stage appropriate expression of the H19 mRNA in the embryonic and postnatal endoderm. In three of five independently derived lines, this transgene is also subject to genomic imprinting, i.e. it is expressed only when inherited through the maternal germline. All three lines with greater than 4 copies of the transgene show maternal specific expression while the two non-parental specific expressing lines represent instances of 2-copy insertions of the transgene. We had also previously shown that the transgene is also imprinted by the criterion that the paternally inherited transgene is hypermethylated in the 4 kb 5' flank. Thus our results support the notion that chromosome specific methylation plays a key role in marking the parental origins of imprinted genes. During this past year we have examined the developmental regulation of methylation of the transgene and demonstrated that the injected DNA contains all signals required for proper control of this process.

2. The *H19* structural gene is required for imprinting of the transgene.

Previously (with S.M. Tilghman at Princeton University) we had generated transgenic lines using DNA constructs where the *H19* structural gene was replaced with the firefly luciferase gene. Examination of the mRNA expression patterns in these lines demonstrates that elements sufficient for correct spatial and temporal expression in endodermal tissues are contained within 800 bp of 5' flanking DNA and 8 kb of 3' flanking DNA. This year we have carefully analyzed expression of these H19-luciferae fusion transgenes as a function of copy number and shown that expression levels are highly position independent. However, we have also shown (at Princeton University) that these transgenes are expressed independently of their parental origin. Likewise, they show the low DNA methylation levels characteristic of the maternally derived endogenous allele.

The loss of imprinting in the H19-luciferase lines may be due either to loss of crucial DNA sequences or to insertion of the foreign luciferase DNA. To distinguish these possibilities, we have microinjected DNAs carrying internal deletions of the *H19* structural gene into mouse zygotes (with S.M. Tilghman at Princeton University). Removal of 701 bp at the 5' end of the gene results in loss of paternal-specific DNA methylation, arguing that it is the loss of DNA sequences that are relevant. The transgenic DNA (like the H19-luciferase transgenes) is inherited from both parents in a relatively unmethylated state. The failure of the transgenes to maintain paternal methylation of their 5' flanks could reflect a failure to methylate the transgenes during gametagenesis or a failure to maintain the methylation during embryogenesis. To discriminate these possibilities, our unit has examined the methylation status of the transgenes during development. We have demonstrated that the transgenic DNA is inherited in the normal relatively hypermethylated state but that this methylation is lost during development supporting the idea that the deleted sequences are required to maintain methylation during the preimplantation period.

Our unit has also analyzed additional transgenic lines generated previously at Princeton University. Expression studies of multiple independent lines demonstrate that sequences encompassing the last 2/3 of the structural gene are not absolutely required for transgene imprinting. Together with the results described above, these studies demonstrate the importance of the first 701 bases of the structural gene for its own regulation. Current studies focus on whether this control element exerts its influence as a cis acting DNA element or as a locally acting regulatory RNA.

3. Upstream elements are also required for imprinting.

Allele specific methylation of the paternally derived *H19* locus has been demonstrated to arise during spermatogenesis and survive during the genome-wide demethylation begun at morula stage. This methylation imprint begins at approximately -5 kb (beyond the boundary of our transgenes) and extends into the structural gene. As described above, we obtained similar results with our wild type transgenes except that sequences within the structural gene itself were not necessarily hypermethylated in transgenic lines. Together, these results suggest that 5' flanking elements may be crucial for genomic imprinting. To test this proposition directly, we have microinjected two additional constructs in order to generate stable transgenic lines. In the first experiment, we have analyzed 4 independent lines that carry an fusion of 1 kb of mouse a-fetoprotein *(AFP)* promoter sequences fused at +26 bp (relative to the *AFP* transcriptional start site) to +3 bp (relative to the transcriptional start site) of the *H19* gene. An additional three lines are also under current analysis. These lines all express the fusion RNA regardless of the germline origin, thus demonstrating the importance of these upstream elements in allele specificity. To further delineate sequences required for imprinting, we are currently analyzing a second construct that carries only 2 kb of 5' flank.

4. Congenic strain construction.

We are interested in isolating novel genes from distal chromosome 7 and in characterizing their expression as to allele specificity. In additional we wish to characterize these genes for their possible roles in Beckwith Wiedemann Syndrome and in Wilms Tumor. These projects are described below. To begin this analysis we have begun the construction of congenic mouse strains that are *domesticus* but carry distal 7 DNA sequences that are *castaneus* in origin. To this end, our unit has identified DNA restriction fragment polymorphisms at *H19* and at *p57KIP2* (currently the most peripheral genes of the imprinted cluster) and have generated F2 mice. We are continuing to backcross distal 7 heterozygote females to domesticus males to generate the congenic strain. Mice homozygous for *castaneus* markers can be generated at any time through brother/sister matings.

Proposed course of the project:

Our future work is aimed at addressing three issues. First, we wish to understand the mechanism for silencing the paternal *H19* allele, i.e. what are the differences in chromosomal structure that allow expression from only the maternal allele of *H19*. Second, we wish to understand the mechanistic relationship between activation of the maternal *H19* allele and silencing of the neighboring insulin genes. Finally, we wish to identify and characterize the expression of other imprinted genes in the region as a means of determining the generality of the mechanisms elucidated for the *H19/Igf-2* gene pair and also as a means of understanding the etiology of Beckwith Wiedemann Syndrome and Wilms Tumor. Experiments to address these issues are outlined below:

1. Mechanism for silencing the paternal H19 allele.

a. Determination of cis acting elements required for imprinting the *H19* transgene and characterization of a bacterial artificial chromosome (BAC) transgenic model system.
Our first experiments are straightforward genetic analyses like those described above. Specifically, we have microinjected additional DNA constructs to determine which upstream elements are required for transgene imprinting as described above. In addition, we are preparing constructs that specifically address the role, if any, for *H19* enhancer elements in allele specific regulation. Our ongoing microinjection experiments utilize derivatives of the 15 kb DNA fragment. As noted above, this DNA directs tissue-, stage-, and allele-appropriate expression when present as multiple copy inserts but not as low copy number inserts. Thus there is a discrepancy with the regulation noted at the endogenous locus. We hypothesize that this is because the 16 kb fragment does not contain all the elements that contribute to the imprinting mark at the chromosomal locus. To address this issue, we have begun to characterize BAC clones that carry both *H19* and *Igf-2* sequences. We will introduce this DNA via microinjection into mouse zygotes derived from our congenic strains. (This will enable us to distinguish endogenous and transgenic DNAs and RNAs). We will then determine the copy number dependence of imprinting of *H19* transcription in this model system. In addition, using these larger BAC clones will allow us to simultaneously determine the effect of mutations at *H19* on expression of the insulin genes.

b. Role of the H19 structural gene in regulation of its own imprinting. Our transgenic analysis indicates that the *H19* structural gene itself (and more specifically the first 701 bp) is crucial for its own imprinting. This requirement is in cis as mutant transgene insertions are not rescued by a wild type H19 at the endogenous locus. However, our experiments do not distinguish whether the structural gene is required as a cis acting regulatory DNA element or if it is required to encode a cis acting regulatory RNA. We are beginning two lines of experiments to distinguish these possibilities. Our first approach utilizes our transgenic model system. We have introduced point mutations into the *H19* promoter to block mRNA synthesis and will microinject these into mouse zygotes. We will first determine that these lines are indeed not expressed in vivo. We will then analyze imprinting of these transgenes using DNA methylation as our assay. If expression of the *H19* gene is required for imprinting, we expect to see low levels of DNA methylation independent of parental origin of the transgene. If expression of the *H19* gene is not required for imprinting, we will expect to see hypermethylation specific to paternally inherited transgenes. The last possibility, hypermethylation of both alleles, will suggest that DNA methylation may be only an effect of lack of expression and not a mechanism for silencing.

Our second approach will utilize a different strategy with distinct assumptions and evaluates whether H19 RNA's interaction with upstream imprinting regulatory elements is required to establish the silent paternal chromosomal organization. We have begun to generate mice with two distinct chromosomal mutations. The first mouse carries lox site mutations flanking upstream elements (-10 to -0.8 kb) previously determined to be required for silencing the paternally inherited H19 transgene. The second mouse will carry lox sites flanking the *H19* structural gene. We will use tissue specific Cre transgenes to generate deletions of these elements and the intervening *H19* sequences at two distinct stages of development: 1) in the testes to generate germline deletion mice and 2) in the fetal liver. We can then determine the temporal requirements for these two elements - upstream regulatory sequences and the *H19* structural gene - in the imprinting process. When these mutations are inherited through the germline, we expect to see loss of imprinting of the *H19* locus, thus confirming our identification of cis acting regulatory elements. (Failure to see such a phenotype will indicate that our transgenic system is not accurately reflecting the chromosomal state. This may be because our transgene, carrying fewer chromosome 7 sequences, does not contain redundant elements that are present in the chromosome. Alternately, the transgene system may not be a real model for chromosomal imprinting. These two possibilities will be evaluated with the BAC transgenes discussed above.) In the second part of the experiment, we will delete each of the two imprinting regulatory elements in fetal liver and evaluate the effect on imprinting. Specifically we will determine if there is a continuous requirement for the presence of these elements or alternatively, if imprinting is stably maintained once established in the germline. If removal of the *H19* structural gene in late embryogenesis does not disrupt imprinting, we will conclude that the mechanistic requirement for these sequences is fundamentally different from that of the upstream elements nd will suggest to us that the RNA may indeed be required to establish imprinting.

2. Mechanistic relationship of *H19* and insulin gene expression: A mechanistic linkage between expression of *H19* and the neighboring *Igf2* and *Ins-2* genes has been established in two ways. First, the genes share a common set of enhancers (at least for endodermal expression) as determined by a deletion of the two *H19* enhancers found at +6 and +7.5 kb. Second, maternal inheritance of a deletion of the *H19* structural gene and 10 kb of upstream sequences results in inappropriate expression of the maternal insulin genes. We have considered two models to account for this mechanistic linkage and to explain the reciprocal imprinting of *H19* and the insulin genes. In the first model, the insulin and *H19* promoters are competing for the common set of enhancers. In the absence of interfering factors, we presume that the *H19* promoter is the superior competitor and interacts with the shared enhancers to the exclusion of the insulin gene promoters. According to this model, the paternal chromosome carries a specific imprint (probably DNA methylation), which blocks the *H19* promoter and thus allows interaction of the enhancers with the insulin genes promoters. In a second model we propose that interaction of the insulin promoters with the shared enhancers is blocked by a boundary element. Removal of the boundary element (as in the deletion described above) or its inactivation by DNA methylation allows interaction of the insulin promoters and the enhancers and thus expression of the insulin genes. By this model, DNA methylation on the paternal chromosome is coincidentally inactivating the boundary element and the *H19* promoter and activation of the insulin genes is not per se dependent upon silencing of the *H19* promoter. This contrasts the first model, in which expression of *H19* and the insulin genes from one chromosome is always excluded.

We will distinguish these two models using knockout mice described above that remove upstream elements required for imprinting of *H19* transgenes. This mutation leaves in tact the *H1* promoter, structural gene, and endodermal enhancers. As described above, we will first determine the *H19* expression phenotype of this mutation when inherited through the male or the female germlines. We expect to see biallelic expression confirming our transgene model system. If we see expression of the paternal *H19* allele we will then assay for expression of maternal and paternal *Igf-2* alleles. If the promoter competition model is correct, we expect to see silencing of the paternal *Igf2* allele. On the other hand, if the boundary element model is correct we expect to see expression of the paternal alleles of both *Igf-2* and *H19*. Further, if we have disrupted the boundary by our deletion we expect to see expression of the maternal *Igf-2* allele.

To date enhancer elements have been identified only for endodermal expression of *H19* and *Igf-2*. We will use deletion analysis of transgenic BAC DNAs to identify enhancers required for expression of these genes in the mesoderm and the choroid plexus. This information will be important in evaluating the way in which genome organization influences gene expression. In mesodermal tissue, *H19* and *Igf-2* are reciprocally imprinted as in endodermal tissue. However, in the choroid plexus, only *H19* is imprinted, while *Igf-2* is biallelically expressed. We are interested in determining the topological relationships of the *Igf-2* and *H19* promoters relative to the endodermal, mesodermal, and choroid plexus enhancers as a possible explanation of the alternate forms of regulation in these tissues.

3. Identification and characterization of other imprinted genes on distal mouse chromosome 7: A striking feature of many imprinted genes is their location in clusters of imprinted loci.

Such a clustering may be coincidental but we are interested in pursuing the notion that it is mechanistically significant. In human medicine, this clustering may play an important role. Beckwith Wiedemann Syndrome and Wilms Tumor has been mapped to the *H19/Igf2* region of human chromosome 11. This mapping is not via the identification of point mutations or deletions but rather because of the association of paternal disomies and /or loss of imprinting at this locus. Thus imprinting diseases like Beckwith or Prader-Willi may be polygenic traits in that they are caused by disruptions in imprinting that affect the gene dosage of several linked genes. To understand this phenomenon better, we intend to characterize the *H19/Igf-2* region and search for new genes in this region. We will then characterize these genes as to their expression patterns and particularly as to their allele specificity. Our aim is to develop a complete transcript map of the region and identification of all imprinted genes.

We have begun to isolate YAC (yeast artificial chromosomes) and BAC clones carrying the mouse *p57KIP2* gene. We will use these clones for two purposes. First, we will generate probes for characterization of the physical linkage of *p57KIP2* and *Mash-2* (the nearest known gene in the imprinting cluster). This characterization may require additional walks with our YAC or BAC clones. Second, we will use these clones as templates for isolation of cDNA clones. We will use three primary methods: exon-trap, cDNA hybridization to YAC (BAC) templates, and identification of homologous human and mouse sequences.

Significance to Biomedical Research and the Program of the Institute:

Genomic imprinting represents an unusual form of gene regulation in which only one allele of a gene is expressed, while the second allele is maintained in a silent or inactive state. The decision of which allele will be silent and which will be active is based solely on the parental origin and is independent of the primary sequence of the alleles. Our recent experiments have indicated that the activity of one gene acts in cis to regulate the activity of its neighbors. Thus in at least these two ways, genomic imprinting represents an excellent and unique model system for addressing the role of chromosomal structure and organization in regulation of gene function.

Genomic imprinting is a crucial aspect of normal mammalian development and disruptions in imprinting are associated with several childhood disorders. Understanding the mechanisms for imprinting will clearly play a role in full comprehension of the etiology of these developmental disorders.

LMGR

LABORATORY OF MOLECULAR GROWTH REGULATION

Bruce H. Howard, M.D., Chief

The Laboratory of Molecular Growth Regulation conducts research on the control of cell proliferation, gene regulation, and DNA replication in eukaryotic cells. In the area of gene regulation, independent groups work in complementary areas, including gene regulation of the developing immune system, control of gene expression during early embryogenesis, gene silencing mediated by epigenetically inherited higher order chromatin domains, RNA polymerase III-dependent transcription of small RNA-encoding genes, and the mechanisms by which gene-specific transcriptional activators interact with the basal RNA polymerase II transcriptional machinery. A collaborative atmosphere is fostered in this laboratory by the overlapping interests of its investigators, as well as by an active schedule of seminars and group meetings.

DEVELOPMENTAL GENE REGULATION OF THE IMMUNE SYSTEM

The Section on Molecular Genetics of Immunity, led by ***Keiko Ozato***, investigates gene regulation in the immune system. Within this framework, the Ozato group analyzes transcription of MHC class I and other genes that are regulated by retinoids and interferons. The primary recent focus has been to elucidate the roles of two transcription factors RXRß and ICSBP. RXRß is a member of the nuclear hormone receptor superfamily which, together with RAR, is involved in retinoid-mediated transcription. ICSBP is a member of the interferon regulatory factor (IRF) family and plays a role in gene regulation mediated by interferons and viruses.

Cooperative biological effects of RAR and RXRß. By studying activities of recently developed synthetic retinoids that are specific for RAR and RXB, the Ozato group showed that RAR ligands (but not RXR ligands) stimulate transcription. However, when the RAR and RXR ligands were added together a strong synergistic activation was noted. Co-addition of the two ligands also led to dramatic increase in neuronal differentiation and apoptosis of EC cells and teratogenic effects in frog and zebrafish development. In vivo footprinting analysis showed that the RXR ligand, although itself had no effect, a combination with an RAR ligand greatly stabilized promoter occupancy *in vivo*. Together, these data show that RXR is an active partner in the RXR/RAR heterodimer and participates in transcription, contrary to the prevailing view that RXR is a silent partner in the heterodimer.

Role of ICSBP in host defense. The in vivo function of ICSBP was studied using ICSBP-/- mice. These mice present a striking abnormality in that myeloid cells are markedly increased from an early stage. Cytokine gene expression was examined with quantitative RT-PCR, which showed that IL-12 expression was markedly reduced in ICSBP-/- mice. IL-12 is a "master" cytokine that controls expression of IFNγin T cells. In accordance these mice were found to be highly susceptible to infection by vaccinia virus and LCMV and toxoplasma. Taken together, the Ozato group's results indicate that ICSBP plays a critical role in host defense.

MOLECULAR REGULATION OF GENE EXPRESSION

RNA polymerase (pol) III is responsible for synthesizing tRNAs, 5S rRNA, U6 snRNA, as well as additional small transcripts such as Alu RNA and Y RNAs. The Unit on Molecular and Cell Biology, led by ***Richard Maraia***, seeks to understand the mechanisms that regulate expression of these RNAs.

Two independent domains of the nuclear autoantigen La control transcription by RNA polymerase III. La protein is an autoimmune antigen that is found associated with precursor tRNAs and other nascent transcripts synthesized by pol III. Some evidence suggests that La protects the 3' ends of nascent transcripts until proper 3'-end maturation occurs. La is a 47 kDa phosphoprotein that participates in the production of these RNAs by facilitating transcription termination and reinitiation by RNA polymerase (pol) III. A previously established assay that monitors the utilization of stable transcription complexes by pol III was used to investigate the ability of various mutants of recombinant La protein to support transcription. RNA binding which is mediated by the N-terminal half of La is required but is not sufficient for efficient transcription activity. A basic region in the C-terminal domain, from amino acids 330-363, rich in glycine and lysine residues, is also required.

Regulation of La's transcription factor activity by small RNAs. Free La protein but not La protein bound to RNA, is active for transcription. A C-terminal deletion mutant of La that exhibits wild type RNA binding activity but no transcription factor activity is able to rescue the RNA-mediated inhibition of wild type La. Additional controls indicate that the inhibitor RNA does not inhibit pol III enzymatic activity directly, rather, La indeed appears to be the target of RNA-mediated inhibition in this system. These data indicate that the RNA-binding domain of La can modulate the activity of stable transcription complexes and suggests a mechanism by which feedback-inhibition of RNA synthesis may be mediated by La.

Regulation of La's transcription factor activity by phosphorylation. A kinase that phosphorylates recombinant La was purified from HeLa cells and identified as casein kinase II (CSII). Deletion and site-directed mutants of La protein indicated that a CSII consensus target site at serine 366 is phosphorylated by the purified kinase. It was found that phosphorylation of S366 by CSII inhibits transcription factor activity of La. Site-directed mutagenesis of S366 to glycine (La S366G) prevented both phosphorylation by CSII and the capacity of CSII treatment to inhibit La transcription factor activity.

Model of La structure and function. The above results support a regulatory role for La in controlling reinitiation by pol III. The transcription factor activity of La can be inhibited by small RNAs by binding to La or by phosphorylation of serine 366. The basic region between residues 330 and 363 of La is required for transcription activity, yet this activity can be inhibited by incorporating phosphate adjacent to this region. Likewise, small RNA can inhibit activity. It is proposed that the RNA binding domain which is located in the N-terminal half of the La protein can present the phosphate backbone of a bound molecule of RNA to the basic region in the C-terminal half of La, thereby inhibiting transcription factor activity. The previously acknowledged two domain structure of La which has been likened to a dumbell, is consistent with this model.

La-associated factors -Ro antigen and Y RNAs. Four different small cytoplasmic (sc)RNAs designated human (h)Y1, hY3, hY4 and hY5 accumulate in human cells as ribonucleoproteins (RNP) associated with the conserved Ro and La autoantigen proteins. The evolutionary conservation of Ro Y RNPs, coupled with their regulated expression and potential involvement in human pathologic conditions, provide evidence to indicate an important biological role for these RNPs, even though no function has yet been ascribed to them. The Maraia group reported the cloning of hY4 RNA gene and, in collaboration with Eric Green (NCHGR), cloning of the hY5 RNA gene. The linear order of the hY RNA genes on chromosome 7q36 was determined to be:
centromere->hY5-hY4-hY3/hY1->telomere.

INITIATION OF DNA REPLICATION IN MAMMALIAN CHROMOSOMES

The key step in regulating the proliferation of cells and viruses is control of initiation of DNA replication. Central to understanding how this control works is understanding the nature of sites where replication begins (i.e. origins of DNA replication). The Section on Eukaryotic DNA Replication, led by ***Mel DePamphilis***, focuses on these problems, as well as how the processes of DNA replication and transcription are turned on at the beginning of mammalian development.

Site-Specific Initiation of DNA Replication in Xenopus Egg Extract Requires Nuclear Structure. DNA replication in the chromosomes of cultured animal cells initiates at specific, heritable sites. The DePamphilis group demonstrated that Xenopus egg extract can initiate DNA replication at specific sites in mammalian chromosomes, but only when the DNA is presented in the form of an intact nucleus. Initiation of DNA synthesis in nuclei isolated from G1-phase Chinese Hamster Ovary (CHO) cells was distinguished from continuation of DNA synthesis at preformed replication forks in S-phase nuclei by a delay that preceded DNA synthesis, a dependence on soluble Xenopus egg factors, sensitivity to a protein kinase inhibitor, and complete labeling of nascent DNA chains. Initiation sites for DNA replication were mapped downstream of the amplified dihydrofolate reductase (DHFR) gene region by hybridizing newly replicated DNA to unique probes and by hybridizing Okazaki fragments to the two individual strands of unique probes. When G1-phase nuclei were prepared by methods that preserved the integrity of the nuclear membrane, Xenopus egg extract initiated replication specifically at or near the origin of bidirectional replication utilized by hamster cells (DHFR ori-β). However, when nuclei were prepared by methods that altered nuclear morphology and damaged the nuclear membrane, preference for initiation at ori-β was significantly reduced or eliminated. Furthermore, site specific initiation was not observed with bare DNA substrates. From these results it was concluded that initiation sites for DNA replication in mammalian cells are established prior to S-phase by some component of nuclear structure, and that these sites can be activated by soluble factors in Xenopus eggs.

DNA Methylation at Mammalian Replication Origins. The *E. coli* replication origin contains numerous DNA methylation sites that play a role in regulating initiation of DNA replication. To ascertain whether DNA methylation might also play a role in mammalian replication origins, the well-characterized DHFR ori-β origin of replication was examined. The two kb region of ori-β that exhibits greatest replication initiation activity also contains numerous potential methylation sites in the form of CpG dinucleotides. Using bisulfite to chemically distinguish cytosine (C) from 5-methylcytosine (mC), all twelve CpGs within the two kb region were found to be methylated, nine of which were located within 356 bp flanking the minimal origin. The density of methylated sites was about 10 times greater than the average for mammalian DNA, consistent with a role for methylation at replication origins.

Regulation of DNA Replication and Gene Expression at the Beginning of Mammalian Development. The DePamphilis group developed a novel approach to identifying cis-acting sequences and trans-acting factors that are required to activate DNA replication and gene expression at the beginning of mammalian development. Plasmid DNA encoding a sensitive reporter gene linked to eukaryotic regulatory sequences is injected into the nuclei of mouse oocytes, fertilized eggs (1-cell embryos) and 2-cell embryos (where zygotic gene activation occurs). The results of such studies have revealed that replication and expression of genes encoded in the injected, extrachromosomal DNA responded to the same signals that regulate these functions in cellular DNA: they required specific cis-acting regulatory sequences and the trans-acting proteins that activate them, and they occurred only when the host cell executed the same function on its own genome. These results, taken together with analyses of endogenous gene expression and results from nuclear transplantation studies, revealed several novel features of zygotic gene expression at the beginning of mammalian development. A time dependent mechanism ("zygotic clock") delays transcription of zygotic genes and translation of nascent mRNA until the 2-cell stage in development. This provides an opportunity to remodel chromatin structure of parental genomes and possibly facilitate genomic imprinting without accidentally exposing genes to premature expression. In addition, the ability to use enhancers to activate promoters or replication origins does not appear until formation of 2-cell embryo, because an enhancer specific co-activator is absent prior to this stage in development. Therefore, the first two S-phases in mouse development occur under conditions where enhancers cannot function, a fact that may contribute to developmental acquisition of site specific initiation of DNA replication. The need for a TATA-box to facilitate promoter activity appears only after cell differentiation occurs and then only as a mediator of enhancer function. Prior to activation of zygotic genes in 2-cell embryos, the activity of promoters and replication origins is repressed by changes in chromatin structure, allowing positive regulatory factors to activate genes selectively. One of the first transcription factors to be expressed at the beginning of mouse development is TEAD2, a member of a new gene family expressed throughout animal development. TEAD2 is expressed specifically during formation of a 2-cell embryo and is able to stimulate strongly the activity of appropriate enhancers, presumably turning on the next cascade of genes in the developmental program.

EUKARYOTIC TRANSCRIPTIONAL REGULATION

The Section on Eukaryotic Transcriptional Regulation, led by ***Yoshiro Nakatani***, endeavors to understand the mechanisms by which: i) gene-specific transcriptional activators interact with the RNA polymerase II transcription machinery to regulate messenger RNA synthesis; and ii) tumor viral proteins perturb normal cellular interactions for transforming cells.

Structural similarity between TAFs and histones. The transcription factor TFIID plays a central role in the assembly of the basic transcriptional machinery into a preinitiation complex. TFIID binds to the core promoter and thus provides a foundation for association of the other initiation factors and RNA polymerase II. Biochemical studies of eukaryotic transcriptional activation demonstrate both physical and functional interactions of activators with TFIID that in turn facilitate preinitiation complex assembly and function. TFIID is a multimeric protein complex, comprising the TATA box binding protein (TBP) and numerous tightly associated factors called TAFs. To date, nine TAF subunits of Drosophila TFIID (dTAFII230, 150, 110, 85, 62, 42, 28a, 28b, 22) have been cloned. In addition, several homologs have been isolated from both human (hTAF) and yeast (yTAF), suggesting that TAF-mediated activation pathways are conserved from yeast to man.

Based on the similarities between dTAF42 and dTAF62, and histones H3 and H4, intermolecular interactions between the histone-like regions in dTAF62 and dTAF42 were examined. The N-terminal histone-like regions of dTAF42 and dTAF62 were expressed independently in E. coli. The stable heteromeric complex was prepared by co-renaturation. The strong interaction between the N-terminal regions of dTAF42 and dTAF62 was also observed in the yeast two hybrid system. However, like histones H3 and H4, neither dTAF42 nor dTAF62 forms a homomeric complex.

In collaboration with Stephen Burley (Rockefeller University), the crystal structure of the N-terminal portions of dTAF42/dTAF62 was determined at 2.0 A resolution. Histone-like regions of TAFs adopt the canonical histone fold, consisting of two short α-helices flanking a long central α-helix. Like histones H3 and H4, dTAF42 and dTAF62 form an intimate heterodimer by extensive hydrophobic contacts between the paired molecules. Moreover, the dTAF42/dTAF62 complex exists as a heterotetramer, resembling the $(H3/H4)_2$ heterotetrameric core of the histone octamer.

Histone octamer-like substructure in TFIID. In related work by the Nakatani group in collaboration with Alan Hinnebusch (Laboratory of Eukaryotic Gene Regulation), the architecture of the TFIID complex was probed by yeast suppressor screening. Several temperature sensitive mutants were isolated by introducing mutations in the histone-like region of yeast TAF60 (yTAF60), a homolog of dTAF62. One of these mutants was used to identify extragenic suppressors from a multicopy plasmid bank. A histone H3-like TAF (yTAF20), a histone H2B-like TAF (yTAF61) and yTAF90, which has WD40-repeats, were isolated as suppressors. Moreover, biochemical studies with human homologs indicate that histone H2B-like TAF forms a homomeric complex which interacts with the histone H3/H4-like TAFs. These results suggest that TFIID contains a histone octamer-like structure composed of two dimers of the histone H2B-like TAF attached to a tetramer of histones H3/H4-like TAFs.

Molecular cloning of P/CAF, a potential tumor suppressor gene. The oncogenic protein E1A, like transforming proteins encoded by adenovirus and several other small DNA tumor viruses, disturbs host cell growth control by interacting with cellular factors that normally function to repress cell proliferation. E1A transforming activity resides in two distinct domains, the targets of which include p300/CBP and products of the retinoblastoma (RB) susceptibility gene family. E1A inhibits the p300/CBP-mediated transcriptional activation of many promoters. Importantly, E1A does not appear to disrupt p300/CBP-activator interactions. This strongly suggested the presence of a cellular counterpart of E1A which binds to p300/CBP and plays an important role in both transcription and cell cycle regulation. Based on these observations and an analogy between species, P/CAF (p300/CBP-associated factor) was cloned.

E1A disturbs P/CAF-p300/CBP interaction
p300/CBP sites important for P/CAF interaction were determined. In vitro interaction study with various p300/CBP mutants led to the conclusion that P/CAF and E1A bind to the same or very closely spaced sites on p300/CBP. This raises the possibility of direct competition between P/CAF and E1A for binding to p300/CBP. As expected, the interaction of P/CAF with the p300/CBP was progressively inhibited by the addition of increasing amounts of E1A. In vivo interaction between P/CAF and p300/CBP was demonstrated by coimmunoprecipitation from cell extracts. Importantly, the endogenous interaction of P/CAF with p300 was drastically inhibited by expression of E1A.

Histone acetylase activity of P/CAF
Acetylation of the N-terminal histone tails has been considered to play a crucial role in accessibility of transcription factors to nucleosomal templates. Based on the information that yGCN5 is a histone acetylase, intrinsic histone acetyltransferase activity in P/CAF was examined. P/CAF acetylated histones H3 and H4, but preferentially H3 in both free histones and the mononucleosome.

CONTROL OF MAMMALIAN CELL PROLIFERATION

The **Human Genetics Section**, under the direction of ***Bruce Howard***, investigates mammalian cell growth regulation, with particular attention to mechanisms underlying cellular immortalization and its converse, cellular senescence.

Histone deacetylases and cell growth control. In previous work, Howard and his coworkers demonstrated that either of two histone deacetylase inhibitors, sodium butyrate or trichostatin A, could dramatically reduce HDF proliferative potential. To pursue these findings further, a putative human histone deacetylase encoding gene, HD2, was isolated and characterized by DNA sequence analysis. Over a 300 amino acid N-terminal conserved region, hHD2 shares 66% identity at the amino acid level with hHD1, the first human histone deacetylase identified. Interestingly, the level of conservation is similar when hHd2 is compared with the Saccharomyces cerevisiae histone deacetylase gene yRPD3.

Cell cycle regulation by P/CAF. In collaborative work, the Howard and Nakatani groups investigated growth regulatory activity of the histone acetyltransferase P/CAF. Exogenous P/CAF expression caused a reproducible redistribution of transfected HeLa cells from S phase to G1 phase, consistent with a role of this gene in growth suppression. Based on evidence that the adenovirus-encoded oncogenic protein E1A and P/CAF compete for binding sites on p300, it was considered that cotransfection of P/CAF with E1A would oppose the mitogenic activity of the E1A. E1A alone had mitogenic activity in HeLa cells, whereas an E1A mutant lacking a p300-binding domain exhibited very weak activity. When P/CAF was cotransfected with E1A, the mitogenic activity of E1A was significantly counteracted by P/CAF. These results support the view that P/CAF and E1A mediate antagonistic effects on cell cycle progression at least in part by competitive binding to the tumor suppressor gene p300.

Chromatin structure in the chromosome 7q subtelomeric region. Histone acetyltransferases and deacetylases are believed to be important determinants of higher order chromatin structure. Consistent with this view, euchromatic and heterochromatic domains can be distinguished by their high and low levels of histone acetylation, respectively. Preparatory to studies on chromatin domain stability in association with cell senescence or immortalization, higher order chromatin structure was mapped over a 1.7 megabase region of the human genome spanning the subtelomeric segment in chromosome 7q. PCR primers which had been designed to define sequence tagged sites (STSs) in the Human Genome Project were utilized to sample chromatin structure at loci arrayed across this region. These experiments revealed the presence of multiple interspersed euchromatic and heterochromatic domains near the chromosome 7q terminus. While the most distal 300 kilobases appeared to be predominantly heterochromatic in nature, euchromatic domains were detected at 200 kilobases from the telomere (corresponding to a CpG island) and at about 25 kilobases from the telomere. No comparable information on higher order chromatin structure near a human chromosome end has been reported previously.

ANNUAL BIBLIOGRAPHY - LMGR FY96

PUBLICATIONS (FY96 = 1995 to 1996), Melvin L. DePamphilis

DePamphilis ML. (ed), DNA Replication in Eukaryotic Cells, Cold Spring Harbor Laboratory Press, NY (1996).

DePamphilis ML. Origins of DNA Replication, pp. 45-86 *In* DNA Replication in Eukaryotic Cells, (M.L. DePamphilis, ed) Cold Spring Harbor Laboratory Press, NY (1996).

DePamphilis ML. Replication Origins in Metazoan Chromosomes, pp. 96-123 *In* Eukaryotic DNA Replication - Frontiers in Molecular Biology (J. Blow, ed), Oxford University Press, England (1996).

DePamphilis ML. Specific Labeling of Newly Replicated DNA, pp. 628-669 *In* Methods in Enzymology - DNA Replication, vol. 262 (J. Campbell, ed.), Academic Press, NY (1995).

Gilbert DM, Miyazawa H, DePamphilis ML. Site-Specific Initiation of Mammalian DNA Replication in *Xenopus* Egg Extract Requires Nuclear Structure, Mol. Cell. Biol. 1995; 15: 2942-2954.

Gilbert DM, Neilson A, Miyazawa, H, DePamphilis ML, Burhans WC. Mimosine Arrests DNA Synthesis at Replication Forks by Inhibiting Deoxyribonucleotide Metabolism, J. Biol. Chem. 1995; 270: 9597-9606.

Henery CC, Miranda M, Wiekowski M, Wilmut I, DePamphilis ML. Repression of Gene Expression At the Beginning of Mouse Development, Devel. Biol. 1995; 169: 448-460.

Majumder S, DePamphilis ML. A Unique Role For Enhancers Is Revealed During Early Mouse Development, BioEssays 1995; 17: 879-889.

Majumder S., Zhao Z., Kaneko K., DePamphilis M.L. Developmental Acquisition of Enhancer Function Requires A Unique Co-Activator Activity, EMBO J. 1996; in press.

Nothias J-Y, Majumder S, Kaneko KJ, DePamphilis ML. Regulation of Gene Expression at the Beginning of Mammalian Development, J. Biol. Chem. 1995; 270: 22077-22080.

Nothias J-Y, Miranda M, DePamphilis ML. Uncoupling Of Transcription And Translation During Zygotic Gene Activation In The Mouse, EMBO J. 1996; 15: 5715-5725.

Rein T, Gärtner U, Niggemann M, DePamphilis ML, Zorbas H. Absence Of An Unusual "Densely Methylated Island" At The Hamster DHFR Ori-b, J. Biol. Chem. 1996; in press.

Rein T, Zorbas H, DePamphilis ML. Active Mammalian Replication Origins Are Associated With A High Density Cluster Of ^{m}CpG Dinucleotides, Mol. Cell. Biol. 1996; in press.

Salas M, Miller JT, Leis J, DePamphilis ML. Mechanisms for Priming DNA Synthesis, pp. 131-176 *In* DNA Replication in Eukaryotic Cells, (M.L. DePamphilis, ed) Cold Spring Harbor Laboratory Press, NY (1996).

PUBLICATIONS (FY96), Bruce H. Howard

Avantaggiati ML, Carbone M, Graessman A, Nakatani Y, Howard BH and Levine AS. The SV40 large T antigen and adenovirus E1a oncoproteins interact with different isoforms of the transcriptional coactivator, p300. EMBO J. 15:2236-2248, 1996.

Englander EW and Howard BH. A naturally occurring $T_{14}A_{11}$ tract blocks nucleosome formation over the human neurofibromatosis type 1 (NF1)-Alu element. J. Biol. Chem. 271(10):5810-5823, 1996.

Howard BH. Replicative senescence: considerations relating to the stability of heterochromatin domains. Exp. Gerontology 31:281-293, 1996.

Yang XJ, Ogryzko VV, Nishikawa J, Howard BH and Nakatani Y. A op300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature 382:319-24, 1996.

Ogryzko VV, Hirai TH, Russanova VR, Barbie DA, Howard BH. Human Fibroblast Commitment to a Senescence-Like State in Response to Histone Deacetylase Inhibitors is Cell Cycle Dependent. Mol. Cell. Biol. 16:5210-18, 1996.

PUBLICATIONS (FY96), Richard J. Maraia

R. J. Maraia. Transcription termination factor La is also an initiation factor for RNA polymerase III, *Proc. Natl. Acad. Sci. USA,* 93;3383-3387, 1996.

R. J. Maraia, Sakulich, A. L., Brinkmann, E. and E. D. Green. Gene for human Ro-associate autoantigen Y5 RNA, *Nucl. Acids Res.* 24;3552-3559, 1996.

D.-Y Chang, Hsu, K. and R.J. Maraia. Monomeric and dimeric Alu RNAs induced by adenovirus assemble into SRP9/14-containing RNPs in HeLa cells, *Nucl. Acids Res.,* 24:4165-4170.

PUBLICATIONS (FY96), Yoshihiro Nakatani

Avantaggiati ML, Carbone M, Graessmann A, Naktani Y, Howard BH, Levine AS. The SV40 large T antigen and adenovirus E1a oncoproteins interact with distinct isoform of the transcriptional co-activator, p300. EMBO J, 1996; 15:2236-48.

Nakatani Y, Bagby S, Ikura M. The histone folds in transcription factor TFIID. J Biol. Chem., 1996; 271:6575-7.

Xie X, Kobuko T, Cohen SL, Mirza UA, Hoffman A, Chait BT, Roeder RG, Nakatani Y and Burley SK. Structural similarity between TAFs and the heterotetrameric core of the histone octamer. Nature 1996; 380:316-22.

Hoffmann A, Chiang CM, Oelgeschläger T, Xie X, Burley SK, Nakatani Y, Roeder RG. A histone octamer-like structure within TFIID. Nature 1996; 380:356-9.

Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y. A p300/CBP-associated factor that competes with the adenoviral E1A oncoprotein. Nature 1996; 382:319-24.

Farmer G, Colgan J, Nakatani Y, Manley JL, Prives C. Functional interaction between p53, the TATA-binding protein (TBP), and TBP-associated factors in vivo. Mol Cell Biol 1996; 16:4295-304.

Nishikawa J, Kokubo K, Horikoshi M, Roeder RG, Nakatani Y. Drosophila $TAF_{II}230$ and the transcriptional activator VP16 competitively bind to the TATA box-binding domain of TBP. Proc. Natl. Acad. Sci. USA, in press.

Ogryzko VV, Schiltz RL, Russanova, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell in press.

Mizzen CA, Yang HJ, Kokubo T, Brownell JE, Bannister AJ, Owen-Hughes T, Workman J, Berger SL, Kouzarides T, Nakatani Y, Allis CD. The $TAF_{II}250$ subunit of TFIID has histone acetyltransferase activity. Cell in press.

PUBLICATIONS (FY96) Keiko Ozato

Blanco, J.C.G., Dey, A., Park, B.-Y., Minucci, S., Jurutka, P.W., Haussler, M.R., and Ozato, K. Inhibition of ligand induced promoter occupancy *in vivo* by a repressor RXRβ. Genes to Cells. 1: 209-221, 1996.

Thornton, A.M., DeVico, A.L., Buller, M.L., Wang, I-M., and Ozato, K. A dominant negative factor of the IRF family converts monocytic cells nonpermissive to HIV and vaccinia virus infection. Proc. Natl. Acad. Sci. USA., 93:383-387, 1996.

Minucci, S., Saint-Jeannet, J.-P., Toyama, R., Scita, G., Taira, M., Levin, A.A., Ozato, K., and Dawid, I.B. Retinoid receptor-selective ligands produce malformations in Xenopus embryos. Proc. Natl. Acad. Sci. USA. 93:1803-1807, 1996.

Minucci, S., Botquin, V., Sylvester, I., Dey, A., Yeom, Y.II., Zand, D.J., Ohbo, K., Ozato, K., and Schöler, H.R. Retinoic acid mediated repression of *Oct3/4* expression coincides with loss of promoter occupancy *in vivo*. EMBO.J., 15:888-899, 1996.

Nelson, N., Kanno, Y., Hong, C., Fujita, T., Fowlkes, B.J., O'Connell, E., Hu-Li, J., Paul, W.E., Jankovic, D., Sher, A.F., Coligan, J.E., Thornton, A., Appella, E., Yang, Y., and Ozato, K.

Expression of Interferon Regulatory Factor (IRF) family proteins in lymphocytes. Induction of Stat 1 and ICSBP expression by T cell activation. J. Immunol., 156: 3711-3720, 1996.

Horn, V., Minucci, S., Orgryzko, V., Adamson, E.D., Howard, B.H., Levin, A.A., and Ozato, K. RAR and RXR Selective Ligands Cooperatively Induce Apoptosis and Neuronal Differentiation in P19 Embryonal Carcinoma Cells. FASEB. J. 1996. 10:1071-1077.

Yoneyama, M., Suhara, W., Fukuhara, Y., Sato, M., Ozato, K., and Fujita, T. Autocrine amplification of type I interferon gene expression mediated by interferon stimulated gene factor 3 (ISGF3). J. Biochem.120: 160-169, 1996.

Hallenbeck, P.L., Minucci, S., Lippoldt, R., Phyillaier, M., Horn, V., Ozato, K., and Nikodern, V.M. Differential 9-cis retinoic acid-dependent transcriptional activation by murine retinoid X receptor α (RXRα) and RXRβ: Role of cell type and RXR domains. J. Biol. Chem. 271:10503-10507. 1996

Nunez, S.B., Medin, J.A., Givel, F., Wahli, W., Ozato, K., and Segars, J.H. The retinoid x receptor and peroxisome proliferator-activated receptor activate estrogen responsive genes independent of the estrogen receptor. J. Cell. Mol. Endocrinol in press.

Thornton, A. M., Ogryzko, V., Dent, A., Sharf, R., Levi, B., Kanno, Y., Staudt, L.M., Howard, B.H. and Ozato, K. A dominant negative mutant of an IRF family protein inhibits both Type I and Type I interferon stimulated gene expression and antiproliferative activity of IFN. J. Immunol (1996). in press.

Wang, I-M., Blanco, J., Tsai, S., Tsai, M-J., O'Malley, B. and Ozato, K. Interferon regulatory Factor-1 (IRF-1) and TFIIB cooperatively enhance interferon-responsive promoter activity in vivo and in vitro. 1996. Mol. Cell. Biol. 16 6313-6324.

Holschke, T., Lohler, J., Kanno, Y., Fehr, T., Giese, N., Rosenbauer, F., Lou, J., Knobeloch, K-P., Gabriele, L., Waring, J., Bachmann, M.F., Zinkernagel, R., Morse, H., Ozato, K., and Horak, I. Immunodeficiency and chronic myelogenous leukemia like syndrome in mice with a targeted mutation of the ICSBP gene. Cell. In press (1996).

REVIEWS, BOOK CHAPTERS

Nunez, S., Medin, J., Keller, H., Wang, K., Ozato, K., Wahli, and Segars, J. Retinoid X receptor β and peroxisome proliferator-activated receptor activate an estrogen response element. Recent Prog. Hormone Res. (In Press), 1996

Kanno, Y., Kanno, T., and Ozato, K. Transcriptional regulation of MHC class I genes -NF-kB and IFN signal transduction pathways (Japanese). Medical Immunology. Kokusai Ishoshuppan Co., Tokyo. 27:297-312, 1994.

Samuel, C.E., and Ozato, K. Induction of interferon and interferon-induced genes. Biotherapy. Cytokine Yearbook (Eds. Pestka, S., and H. Schellekens). Kluwer Academic Publisher (Dordrecht, the Netherlands). 8:183-187, 1996.

Dey, A and Ozato, K. Genomic footprinting of retinoic acid regulated promoters in embryonal carcinoma cells. Methods (Academic press). Ed: Zaret, K. In Press.

Minucci, S. and Ozato, K. Retinoid receptors in transcriptional regulation. Curr. Op. Gen. Dev. 1996.

Project Numbers - Annual Report 1996

LABORATORY OF MOLECULAR GROWTH REGULATIONS (LMGR)

Z01 HD 00412-09	Molecular Regulation of Gene Expression Richard J. Maraia, M.D.
Z01 HD 00505-03	Transcription and Cell Cycle Regulation Yoshihiro Nakatani, Ph.D.
Z01 HD 00506-01	Initiation of DNA Replication in Mammalian Chromosomes Melvin L. DePamphilis, Ph.D.
Z01 HD 00507-01	Regulation of DNA Replication and Gene Expression at the Beginning of Mammalian Development Melvin L. DePamphilis, Ph.D.
Z01 HD 01310-10	Developmental Gene Regulation of the Immune System Keiko Ozato, Ph.D.
Z01 HD 08719-16	Development and Uses of Eukaryotic Vectors Bruce H. Howard, M.D.

ZO1 HD 00508-599

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 0041209 LMGR

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Regulation of Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Richard J. Maraia	Senior Research Investigator	LMGR, NICHD
Others:	Dau-Yin Chang	Intra Personnel Act	Univ. MD/LMGR, NICHD
	John Goodier	Visiting Fellow	LMGR, NICHD
	Amy Lynn Sakulich	Biologist	LMGR,NICHD
	Hao Fan	Visiting Fellow	LMGR,NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Growth Regulation

SECTION

Unit on Molecular & Cell Bilology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.75	2.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c)x Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Research on small RNA gene expression was continued. RNA polymerase (pol) III is responsible for synthesizing tRNAs, 5S rRNA, U6 snRNA and other small transcripts such as Alu RNA. Regulation of pol III reinitiation by the human autoimmuneantigen La was investigated. Alu sequences are small, mobile genetic elements endogenous to the human genome whose RNA-mediated transposition causes insertional mutagenesis in humans. Investigation of Alu retroposon structure and interaction with the Alu RNA binding protein SRP9/14 was continued. This revealed sequences in Alu RNA that severely affect SRP9/14 binding and probably determine retroposon competence.

Summary of Major Findings:

1. A model to explain the course of Alu amplification in primates was developed from findings on interactions between Alu RNA and the Alu RNA-binding ptotein SRP9/14. Alu RNA structure, SRP9/14 binding, and expression in vivo of reconstructed Alu retroposons that were differentially active through primate evolution were analyzed. This revealed that human Alu retroposons have lost a high affinity binding site for SRP9/14 in the right monomer of the dimeric Alu element. This was accompanied by production of Alu left monomer RNA and a decline in the Alu amplification rate that had been noted previously in the human lineage (as compared to primitive primates). This provides the first evidence that points to the Alu sequence itself, as opposed to putative control regions in flanking DNA, as a major determinant of Alu amplification in modern primates and humans.
2. The human autoimmune antigen/phosphoprotein known as La is a transcription termination factor. La controls reinitiation of small RNA genes by also functioning as an (re)initiation cofactor for RNA pol III. The structural regions responsible for La's transcription factor activity were mapped to RRM-2 and a basic region near the C-terminus. RNA concentration-dependent inhibition of this activity in vitro provides a mechanism by which feedback regulation may control small RNA synthesis in vivo. A kinase was purified from HeLa cells that phosphorylates La at a single site adjacent to the C-terminal basic region; this phosphorylation inhibits La's transcription factor activity but not RNA binding. Prelimninary evidence is consistent with phosphorylation of this site in vivo.

Project Description

Objectives:

To understand the mechanisms that regulate small RNA expression including Alu retroposons.

Methods Employed:

Tissue culture (TC) cells were used preparatively as a source of nuclear extract for *in vitro* transcription and RNA-binding protein. Protein purification was by ion-exchange, affinity, and gel filtration chromatography. Isolation and analysis of active RNA polymerase III-containing transcription complexes utilized biotin-streptavidin-based solid state methods. In vitro mutagenesis followed by affinity purification of "tagged" recombinant proteins produced in bacteria was used to generate mutant protein for analysis by transcription reinitiation assays and RNA-mediated electrophoretic mobility shift assay (EMSA). Yeast were transformed with mammalian class III genes. Mammalian RNA-binding proteins were overexpressed in yeast. cDNA was constructed from HeLa or owl monkey poly(A)+ RNA and isolated by PCR. Human gene isolation was done by first screening yeast artificial chromosome (YAC) libraries. PCR-based methods were also used for site directed mutagenesis and generation of DNA templates (and mutants thereof) for T7 RNA polymerase-directed *in-vitro* RNA synthesis. RNA and ribonucleoprotein (RNP) analyses were also by primer extension and immunoprecipitation. RNA structural analyses was by native and denaturing gel electrophoresis, computer-assisted free energy, and phylogenetic modeling based on phlyogenetic comparative analysis. Immunoblot and immunoprecipitation were used. Dissociation constant determinations of interactions between pure RNA and pure proteins was by EMSA.

Major Findings:

The decline in human Alu transposition was accompanied by a decrease in SRP9/14 binding and alterations in Alu RNA metabolism Nearly one million copies of Alu repetitive elements exist in human DNA although most of these were inserted during early primate evolution during a period when the Alu RNA-binding protein, SRP9/14, was deregulated and underwent structural changes. Alu insertions indeed occur in humans, albeit at a relatively low rate, by a retrotransposition process in which dimeric Alu RNA is a presumed intermediate. In cells, Alu dimeric transcripts are few and labile, giving rise to stable, left monomeric, scAlu RNAs by RNA 3'-processing. We constructed the three Alu consensus sequences that represent the Alu retroposons that were active during ancient, intermediate and modern evolutionary times. We showed that the Alu right monomer has successively accumulated mutations in its SRP9/14 binding sites as the Alu retroposon evolved in higher primates and humans. Differences in predicted structures of the Alu retroposon sequences were associated with 13% change in minimal free energy despite only 4% difference in sequence. Moreover, these significant structural differences occurred in three SRP9/14 binding sites resulting in a progressive decrease in affinity of the right monomers for SRP9/14 as the Alu sequence evolved. Structural differences between right monomers were confirmed by comparing their mobilities in native and denaturing gels. In sharp contrast to the Alu right monomer, scAlu RNA derived from the Alu left monomer has remained completely unchangeed in its SRP9/14 binding sites through the same primate evolutionary periods providing strong evidence to suggest that the ability of scAlu RNA to interact with SRP9/14 has been activiely selected for by modern primates. In vivo, the asymmetry of left and right monomer affinity for SRP9/14 leads to scAlu RNA production by modern Alu retroposons but not by the reconstructed Alu retroposons that were active in ancient and intermediate times. Thus, a decrease in the affinity between SRP9/14 and the Alu right monomer somehow facilitates scAlu RNA accumulation in vivo, probably at the level of production. Moreover, binding affinity between Alu right monomer RNA and SRP9/14 is inversely correlated with Alu retroposition rates. Put another way, 3'-end processing of Alu RNA was associated with the low rate of Alu transposition in the human lineage. For example, the lowest affinity observed is between human-specific Alu right monomer RNA and SRP9/14 and this is correlated with the low rate of Alu transposition that has previously been noted in the human lineage. These observations provide insight into how retroposons may coevolve with trans-acting factors in complex dynamic genomes and also prompt a model of Alu amplification in primates.

Two independent domains of the nuclear autoantigen La control transcription by RNA polymerase III. La protein is an autoimmune antigen that is found associated with precursor tRNAs and other nascent transcripts synthesized by pol III. Some evidence suggests that La protects the 3' ends of nascent transcripts until proper 3'-end maturation occurs. La is a 47 kDa phosphoprotein that participates in the production of these RNAs by facilitating transcription termination and reinitiation by RNA polymerase (pol) III. A previously established assay that monitors the utilization of stable transcription complexes by pol III was used to investigate the ability of various mutants of recombinant La protein to support transcription. RNA binding which is mediated by the N-terminal half of La is required but is not sufficient for efficient transcription activity. A basic region in the C-terminal domain, from amino acids 330-363, rich in glycine and lysine residues, is also required.

Regulation of La's transcription factor activity by small RNAs. Free La protein but not La protein bound to RNA, is active for transcription. A C-terminal deletion mutant of La that exhibits wild type RNA binding activity but no transcription factor activity is able to rescue the RNA-mediated inhibition of wild type La. Additional controls indicate that the inhibitor RNA does not inhibit pol III enzymatic activity directly, rather, La indeed appears to be the target of RNA-mediated inhibition in this system. These data indicate that the RNA-binding domain of La can modulate the activity of stable transcription complexes and suggests a mechanism by which feedback-inhibition of RNA synthesis may be mediated by La.

Regulation of La's transcription factor activity by phosphorylation. A kinase that phosphorylates recombinant La was purified from HeLa cells by conventional chromatography. The biochemical characteristics of this kinase were determined using various kinase inhibitors and $[^{32}P\text{-}\gamma]$ATP versus $[^{32}P\text{-}\gamma]$GTP donors. The analysis suggested that the activity was due to casein kinase II (CSII). Standard antiserum to the alpha subunit of CSII detected a band of the correct size on Western blots that copurified with the kinase activty. Deletion mutants of La protein suggested that a CSII consensus target site at serine 366 was phosphorylated by the purified kinase. Commercially available CSII gave the same pattern of phosphorylation of mutabnt La proteins as did the purified kinase. Site-directed mutagenesis of S366 confirmed that this site was used by the purified and commercially available CSII. Thus, CSII was purified from HeLa cell extract as a kinase that phosphorylates La. We found that phosphorylation of S366 by CSII inhibits transcription factor activity of La. Site-directed mutagenesis of S366 to glycine prevented phosphorylation by CSII. Moreover, CSII treatment did not inhibit transcription factor activity of La S366G. Attempts to determine whether serine 366 is phosphorylated in vivo are underway; preliminary evidence suggests that it is.

Model of La structure and function. The above results support a regulatory role for La in controlling reinitiation by pol III. The transcription factor activity of La can be inhibited by small RNAs by binding to La or by phosphorylation of serine 366. The basic region between residues 330 and 363 of La is required for transcription activity, yet this activity can be inhibited by incorporating phosphate adjacent to this region. Likewise, small RNA can inhibit activity. We propose that the RNA binding domain which is located in the N-terminal half of the La protein can present the phosphate backbone of a bound molecule of RNA to the basic region in the C-terminal half of La, thereby inhibiting transcription factor activity. The previously acknowledged two domian structure of La which has been likened to a dumbell, is consistent with this model.

Control of the use of stable transcription complexes as opposed to their assembly. A regulatory role for La in transcription implies a novel mode of transcriptional regulation for class III genes. Although assembly of transcription complexes is the rate limiting step for transcription initiation, once formed, these complexes are stable and can undergo multiple rounds of RNA synthesis by pol III (2, 9, 11). Previously, examples of regulation of class III gene transcription involved modulation of the amount or the activity of transcription complex assembly factors. By contrast to the intense study of the process of transcription complex assembly, much less attention has been focused on factors that control the efficient recycling of stable transcription complexes by pol III (5, 6, 10, 13, 16). Immobilized transcription complexes assembled in unfractionated extract can be isolated and used to examine factor requirements for efficient recycling by pol III (13, 16). Perhaps additional factors related to La also can modulate transcription rate by controling the use of transcription complexes by pol III.

La-associated factors -Ro antigen and Y RNAs. Four different small cytoplasmic (sc)RNAs designated human (h)Y1, hY3, hY4 and hY5 accumulate in human cells as ribonucleoproteins (RNP) associated with the conserved Ro and La autoantigen proteins (7, 24). *In vivo* Y RNAs are most abundant in heart and brain tissues (see ref. (25), and this pattern is established during fetal development (UMCB, unpublished data). The presence of autoimmune antibodies directed against Ro RNPs is tightly associated with the development of congenital complete heart block in infants born to anti-Ro positive mothers whose autoantibodies cross the placenta (22). Furthermore, it has been reported that these anti-Ro autoantibodies are localized to the affected infants' cardiac conduction system, and in experimental systems inhibit cardiac cell membrane repolarization (1, 4) and refs. therein). Although initially identified as human autoantigens Ro and La Y RNPs have since been found in several species including *Xenopus* and *c. elegans* (8, 12, 20, 21, 23). Unlike most pol III synthesized RNAs, a subset of mammalian Y RNAs retain their oligo(U) 3' termini and remain associated with the La antigen, although the significance of this is unknown. Nonetheless, in this regard Y RNAs are similar to the adenovirus encoded VA_I and VA_{II} RNAs and the Epstein Barr virus small RNAs which are synthesized by pol III and remain complexed with La. Although each of these virally-encoded class III templates produces RNAs that inhibit the double strand-activated kinase which regulates eIF2α the function of Y RNAs remains unknown. The evolutionary conservation of Ro Y RNPs coupled with their regulated expression and involvement in human pathologic conditions which are manifest in development provide ample evidence to indicate an important biological role for these RNPs even though no function has yet been ascribed to them (7, 8, 12, 18, 20-22, 24). Little is known about their expression even though Y RNAs unlike most other class III genes appear to represent tissue-selective products.

The first Y RNA genes cloned were hY1 and hY3 both of which reside within 4 kb of each other (24). However, further attempts by several groups to clone mammalian Y RNA genes have been impeded by the presence of an abundance of dispersed Y pseudogenes in mammalian genomes (3, 19, 21). The UMCB reported the cloning of hY4 RNA gene (17). Recently, and again in collaboration with Eric Green (NCHGR), the remaining human Y RNA gene, hY5 was cloned. Like hY4, the hY5 scRNA gene is also dependent on its upstream control elements for transcription in transfected cells (14, 17).

The linear order of the hY RNA genes on chromosome 7q36 was determined to be:
centromere->hY5-hY4-hY3/hY1->telomere (hY3 and hY1 genes are within 4 kb of each other and their orientation relative to each other has not yet been determined). This gene family appears to represent a unique locus of upstream-regulated class III genes that are differentially regulated. Investigation of the regulation of this gene family is expected to reveal novel mechanisms of pol III regulation.

Development of HeLa cell fractionated extract for in vitro transcription. Until recently, the UMCB's studies on transcription by pol III have for historical reasons, used nuclear extract derived from murine cells. However, the laboratories who lead this field use HeLa cell-derived extract that has been seperated into fractions TFIIIA, TFIIIB, and TFIIIC. It is therefore exciting to report that the UMCB has successfully seperated HeLa cell extract into these fractions and can reconstitute transcription activity efficiently. Significantly, TFIIIC has been seperated into TFIIIC1 and TFIIIC2 fractions and these are differentially used by class III genes that use internal and external promoter structures, reproducing the results obtained in the Roeder laboratory. Thus, we now have a fractionated extract system that is standard in other laboratories. Characterization of these fractions indicates that both the TFIIIB fraction and our pol III preparation contain small amounts of La antigen.

The fractionated extract for in vitro transcription will also be used for examination of the pol III-associated activity that processes 3' ends of nascent transcripts. It is important to study processing of pol III-synthesized RNA as opposed to purified RNA because it was previously shown that differential 3' processing is coupled to transcription by pol III (15). Highly purified TFIIIC1 and TFIIIC2 in combination with TFIIIB was used in standard (i.e., soluble) transcription reactions to examine expression of a human $tRNA_i^{met}$ gene. Preliminary experiments indicate that addition of purified La stimulates transcription and profoundly affects processing of human pre-$tRNA_i^{met}$. Thus, this fractionated system confirms and extends our previous results and should lend itself well to dissection of the relationship between transcription initiation, termination and 3' processing activity.

Significance to biomedical research and the program of the Institute.

The impact of Alu SINE amplification on the human genome has been substantial. Germ line Alu mobility has caused recognizable hereditary disorders in humans by mechanisms that are modelled but poorly understood. Alu transcripts mediate Alu retrotransposition yet factors involved have not previously been identified. The primate SRP14-Alu RBP is significantly larger than its mammalian counterrparts and is overexpressed specifically in primates. SRP14-Alu RBP modulates Alu RNA levels in vivo and appears to have been involved in the primate-specific amplification of Alu elements which shaped the human genome. Thus, this protein is an excellent candidate factor involved in mediating Alu transposition; however an experimental system for studying this is still not yet available. In addition, many human mRNAs contain Alu sequences in their untranslated regions; an unpredictably high percentage of these are related to immune and or cell recognition suggesting that Alu elements have contributed to their functional genetic variability. The facts that SRP14 is involved in modulating translation of secreted proteins and that Alu RNPs increase dramatically in adenovirus-infected cells suggests an independent role for Alu in response to viral infection.

Pol III must produce very high levels of tRNAs and 5S rRNAs required for protein synthesis. Understanding the factors, mechanisms and regulation of the efficient use of limited amounts of pol III in cells is our goal. Elucidation of regulated physiologic connections between pol III activity, protein synthesis and DNA replication should benefit our understanding of basic aspects of cellular function.

Y RNA gene function in humans (or any species) is completely unknown yet hY RNA-containing Ro RNPs are targetted by autoimmune disorders of adults and infants. Cloning and functional characterization of the hY4 and hY5 RNA genes which each produce pol III transcript constituents of scRNPs was accomplished.

These advances were directed toward achieving the goals of the program of the institute.

Proposed Course

1. Continue investigation of regulation of class III gene expression by the phosphoprotein La. Specifically,

2. Continue to examine the relationship between SRP9/14, Alu RNA binding and retroposition rate. Specifically, extend our investigations on the relationship between different Alu retroposon sequences and SRP9/14 to the effects of different SRP9/14 proteins (i.e., variable C-terminal tails) on affinity for Alu RNA and expression of Alu RNA in vivo.

Publications

Maraia, RJ Transcription termination factor La is also an initiation factor for RNA polymerase III,*Proc. Natl. Acad. Sci. USA,* 199693:3383-3387.

Maraia, RJ, Sakulich, AL, Brinkmann, E, Green, ED. Gene for human Ro-associated autoantigen Y5 RNA, *Nucl. Acids Res.*, in press.

References

1. Alexander, E., J. P. Buyon, T. T. Provost, and T. Guarnieri. 1992. Anti-Ro/SS-A antibodies in the pathophysiology of congenital heart block in neonatal lupus syndrome, an experimental model. Arthritis & Rheumatism 35:176-189.

2. Bogenhagen, D. F., W. M. Wormington, and D. D. Brown. 1982. Stable transcription complexes of Xenopus 5S RNA genes: a means to maintain the differentiated state. Cell 28:413-421.

3. Crouch, D., and E. H. H. Liebke. 1989. The molecular cloning of a mouse Ro RNA, mY1-like sequence. Nucl. Acids Res. 17:4890.

4. Deng, J. S., L. W. J. Bair, S. Shen-Schwarz, R. Ramsey-Goldman, and T. J. Medsger. 1987. Localization of Ro (SS-A) antigen in the cardiac conduction system. Arthritis Rheum 30:1232-1238.

5. Dieci, G., L. Duimio, F. Coda-Zabetta, K. U. Sprague, and S. Ottonello. 1993. A novel RNA polymerase III transcription factor fraction that is not required for template commitment. J. Biol. Chem. 268:11199-11207.

6. Dieci, G., and A. Sentenac. 1996. Facilitated recycling by RNA polymerase III. Cell 84:245-252.

7. Hendrick, J. P., S. L. Wolin, J. Rinke, M. R. Lerner, and J. A. Steitz. 1981. Ro small cytoplasmic ribonucleoproteins are a subclass of La ribonucleoproteins: further characterization of the Ro and La small ribonucleoproteins from uninfected mammalian cells. Mol. Cell. Biol. 1:1138-1149.

8. Itoh, Y., J. D. Kriet, and M. Reichlin. 1990. Organ distribution of the Ro (SS-A) antigen in the guinea pig. Arthritis & Rheumatism 33:1815-1821.

9. Kassavetis, G. A., B. R. Braun, L. H. Nguyen, and E. P. Geiduschek. 1990. S. cerevisiae TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell 60:235-245.

10. Kovelman, R., and R. G. Roeder. 1990. Sarkosyl defines three intermediate steps in transcription initiation by RNA polymerase III: application to stimulation of transcription by E1A. Genes & Dev. 4:646-658.

11. Lassar, A. B., P. L. Martin, and R. G. Roeder. 1983. Transcription of class III genes: formation of preinitiation complexes. Science 222:740-748.

12. Lerner, M. R., J. A. Boyle, J. A. Hardin, and J. A. Steitz. 1981. Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. Science 211:400-402.

13. Maraia, R. J. 1996. Transcription termination factor La is also an initiation factor for RNA polymerase III. Proc. Natl. Acad. Sci., USA 93:3383-3387.

14. Maraia, R. J., E. Brinkman, A. L. Sakulich, and E. D. Green. 1995. The gene encoding human Ro-associated autoantigen hY5 RNA. Nucl Acids Res, In press

15. Maraia, R. J., D.-Y. Chang, A. P. Wolffe, R. L. Vorce, and K. Hsu. 1992. The RNA polymerase III terminator used by a B1-Alu element can modulate 3' processing of the intermediate RNA product. Mol. Cell Biol. 12:1500-1506.

16. Maraia, R. J., D. J. Kenan, and J. D. Keene. 1994. Eukaryotic transcription termination factor La mediates transcript release and facilitates reinitiation by RNA polymerase III. Mol. Cell. Biol. 14:2147-2158.

17. Maraia, R. J., N. Sasaki-Tozawa, C. T. Driscoll, E. D. Green, and G. J. Darlington. 1994. The human Y4 small cytoplasmic RNA gene is controlled by upstream elements and resides on chromosome 7 with all other hY scRNA genes. Nucl Acids Res 22:3045-3052.

18. O'Brien, C. A., and J. B. Harley. 1990. A subset of hY RNAs is associated with erythrocyte Ro ribonucleoproteins. EMBO J. 9:3683-3689.

19. O'Brien, C. A., and J. B. Harley. 1992. Association of *hY4* pseudogenes with *Alu* repeats and abundance of hY RNA-like sequences in the human genome. Gene 116:285-289.

20. O'Brien, C. A., K. Margelot, and S. L. Wolin. 1993. *Xenopus* Ro ribonucleoproteins: members of an evolutionarily conserved class of cytoplasmic ribonucleoproteins. Proc. Natl. Acad. Sci. USA 90:7250-7254.

21. Pruijn, G. J. M., P. A. E. T. M. Wingens, S. L. M. Peters, J. P. H. Thijssen, and W. J. van Venrooij. 1993. Ro RNP associated Y RNAs are highly conserved among mammals. Biochim et Biophys Acta 1216: 395-401.

22. Taylor, P. V., J. S. Scott, L. M. Gerlis, F. R. C. Path, E. Esscher, and O. Scott. 1986. Maternal antibodies against fetal cardiac antigens in congenital complete heart block. New England Journal of Medicine 315:667-672.

23. Van Horn, D. J., D. Eisenberg, C. A. O'Brien, and S. L. Wolin. 1995. *Caenorhabditis elegans* embryos contain only one major species of Ro RNP. RNA 1:293-303.

24. Wolin, S. L., and J. A. Steitz. 1983. Genes for two small cytoplasmic Ro RNAs are adjacent and appear to be single-copy in the human genome. Cell 32:735-744.

25. Wolin, S. L., and J. A. Steitz. 1984. The Ro small cytoplasmic ribonucleoproteins: identification of the antigenic protein and its binding site on the Ro RNAs. Proc. Natl. Acad. Sci. 81:1996-2000.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00505-03 LMGR

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transcription and Cell Cycle Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. :	Yoshihiro Nakatani	Visiting Scientist	LMGR, NICHD
Other:	Ho, Chi-Yip	Visiting Fellow	LMGR, NICHD
	Swanson, Mark	IRTA	LMGR, NICHD
	Schiltz, Lou	NRC	LMGR, NICHD
	Vassilev, Alex	Visiting Fellow	LMGR, NICHD
	Yang, Xiang-Jiao	Visiting Associate	LMGR, NICHD

COOPERATING UNITS *(if any)*

NICHD, NIH (Bruce H. Howard)
Rockefeller University (Robert G. Roeder)
Rockefeller University (Stephen K. Burley)
NICHD, NIH (Alan G. Hinnebusch)

LAB/BRANCH

Laboratory of Molecular Growth Regulation

SECTION

Unit on Eukaryotic Transcriptional Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.4	5.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Although mechanisms by which RNA polymerase II initiates transcription from the repressive chromatin is not clear, transcription initiation factor TFIID must have access to the core promoter on the chromatin. TFIID is the only factor which can bind by itself to the core promoter among the members of the preinitiation complex, and it builds a platform for the preinitiation complex. TFIID is a large multisubunit complex, consisting of the TATA-box binding protein (TBP) and a number of tightly associated subunits (TAFs). We observed that the N-terminal regions of Drosophila TAF42 (dTAF42) and TAF62 (dTAF62) have sequence similarities with the C-terminal core regions of histones H3 and H4, respectively. The histone-homologous regions of dTAF42 and dTAF62 form a heteromeric complex. However, neither dTAF42 nor dTAF62 forms a homomeric complex, in agreement with a nucleosomal histone character. The X-ray crystal structure of the dTAF42/dTAF62 complex indicates that TFIID contains a structure closely resembling that of the (H3/H4)2 heteromeric core of the histone octamer. Moreover, yeast suppressor screening and biochemical analyses suggest that TFIID contains a histone octamer-like structure composed of two dimers of the histone H2B-like TAF attached to a tetramer of histone H3/H4-like TAFs.

Adenovirus can perturb the cell cycle by binding to p300 and CBP, which regulate transcription of the different genes required as the cycle progresses. We determined that a novel cellular factor (P/CAF) having intrinsic histone acetylase activity is associated with p300/CBP and competes with E1A for access to it. E1A can disrupt the P/CAF-p300/CBP complex, and may exert many of its effects on normal cellular function by this route. Conversely, overexpression of P/CAF in cultured cells inhibits the cell cycle and counteracts the activities of E1A.

Project Description

Objectives

To understand the mechanisms by which 1) gene-specific transcriptional activators interact with the RNA polymerase II transcription machinery to regulate messenger RNA synthesis; and 2) tumor viral proteins perturb normal cellular interactions for transforming cells.

1. Mechanism of Transcriptional activation

Introduction

The transcription factor TFIID plays a central role in the assembly of the basic transcriptional machinery into a preinitiation complex. TFIID binds to the core promoter and thus provides a foundation for association of the other initiation factors and RNA polymerase II. Biochemical studies of eukaryotic transcriptional activation demonstrate both physical and functional interactions of activators with TFIID that in turn facilitate preinitiation complex assembly and function. TFIID is a multimeric protein complex, comprising the TATA box binding protein (TBP) and numerous tightly associated factors called TAFs. To date, nine TAF subunits of Drosophila TFIID ($dTAF_{II}230$, 150, 110, 85, 62, 42, 28α, 28β, 22) have been cloned. In addition, several homologs have been isolated from both human (hTAF) and yeast (yTAF), suggesting that TAF-mediated activation pathways are conserved from yeast to man.

A central question in eukaryotic transcriptional regulation is how TFIID gains access to a chromatin template, and how stable association is maintained within the chromosome. A potentially relevant finding is that the N-terminal regions of the dTAF42 and dTAF62 proteins have sequence similarities with the C-terminal core domain of histones H3 and H4, respectively.

Major Findings

dTAF42/dTAF62 histone-like regions form a heteromeric complex

Based on the similarities between dTAF42 and dTAF62, and histones H3 and H4, intermolecular interactions between the histone-like regions in dTAF62 and dTAF42 were examined. The N-terminal histone-like regions of dTAF42 and dTAF62 were expressed independently in E. coli. The stable heteromeric complex was prepared by co-renaturation. The strong interaction between the N-terminal regions of dTAF42 and dTAF62 was also observed in the yeast two hybrid system. However, like histones H3 and H4, neither dTAF42 nor dTAF62 forms a homomeric complex.

Structural similarity between TAFs and histones (in collaboration with Stephen K. Burley)

The crystal structure of the N-terminal portions of dTAF42/dTAF62 was determined at 2.0 A resolution (Fig. 1). Histone-like regions of TAFs adopt the canonical histone fold, consisting of two short α-helices flanking a long central α-helix. Like histones H3 and H4, dTAF42 and dTAF62 form an intimate heterodimer by extensive hydrophobic contacts between the paired molecules. Moreover, the dTAF42/dTAF62 complex exists as a heterotetramer, resembling the $(H3/H4)_2$ heterotetrameric core of the histone octamer.

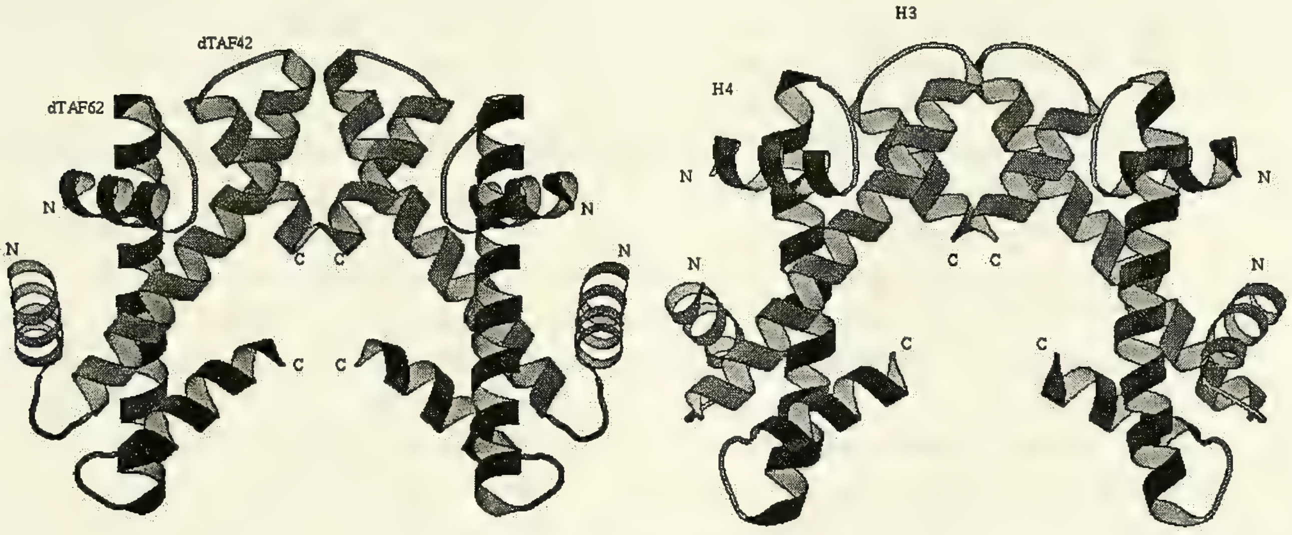


Fig. 1 Crystal structures of the dTAF42/dTAF62 (left) and the histones H3/H4 (right) heterotetrameric complexes.

Histone octamer-like substructure in TFIID (in collaboration with Alan G Hinnebusch and Robert G. Roeder)

We have attempted to understand the architecture of the TFIID complex by yeast suppressor screening. Several temperature sensitive mutants were isolated by introducing mutations in the histone-like region of yeast TAF60 (yTAF60), a homolog of dTAF62. One of these mutants was used to identify extragenic suppressors from a multicopy plasmid bank. A histone H3-like TAF (yTAF20), a histone H2B-like TAF (yTAF61) and yTAF90, which has WD40-repeats, were isolated as suppressors. Moreover, biochemical studies with human homologs indicate that histone H2B-like TAF forms a homomeric complex which interacts with the histone H3/H4-like TAFs. These results suggest that TFIID contains a histone octamer-like structure composed of two dimers of the histone H2B-like TAF attached to a tetramer of histones H3/H4-like TAFs.

Perspectives

In some promoters, including the adenovirus major late and human gfa promoters, the TFIID footprint extends from the TATA box through to 35 bp downstream from the initiation site, whereas the TBP footprint extends only over the TATA box region. These results indicate that one or more TAFs contribute to the downstream interaction. Recent studies with recombinant TAFs demonstrated that dTAFII150 alone can bind to the initiator element. Moreover, the dTAFII150-TBP complex gives a footprint from the TATA box through to 35 bp downstream from the initiation site, like native TFIID. However, the dTAFII150-TBP complex does not produce DNaseI hyper-sensitive sites as observed for native TFIID. Thus, it is likely that other TAFs are also involved in the downstream interaction. We predict that the histone octamer-like substructure of TFIID is bound to the downstream sequence given that, similar to histone core, TFIID produces DNaseI hyper-sensitive sites about every 10 bp.

The downstream interaction plays an important role in both basal and activated transcription. TFIID binds to the downstream region in a DNA sequence-dependent fashion, at least in the human gfa promoter, contributing to both stable TFIID binding and effective transcription in vitro. In contrast, in the adenovirus E4 promoter, TFIID does not bind to the downstream region and produces very low basal transcription activity. Importantly, activators induce the downstream interaction and facilitate preinitiation complex formation. Furthermore, the histone-like domains may have a more critical role in a natural chromatin environment, e. g., stimulation of the displacement of nucleosomal histones near the transcription start site and/or maintenance of histone exclusion from the transcription start site.

Proposed Course

To test the function of the histone-like substructure in TFIID, we prepared a mini TFIID complex composed of dTBP (dTAF42/dTAF62)$_2$. We are currently testing whether this mini-complex can interact with the downstream sequences of the core promoter like a complete TFIID. Given that histone-like TAFs are identified as targets for various activators including p53 and VP16, we are planing to test whether the mini-TFIID complex can respond to these activators in a reconstituted transcription system in vitro. If it is positive, we will prepare similar complexes with various mutant TAFs to understand the mechanism of transcriptional activation.

To determine the functions of TAFs in activated transcription in vivo, we are attempting to isolate yeast mutants which cannot support normal response to transcriptional activators by introducing random mutagenesis in the various yeast TAFs. Isolating mutants will allow us to determine how TAF mutations altered activator response by analyzing purified TFIID from mutants.

2. Mechanism of cell transformation by tumor virus

Introduction

The transforming proteins encoded by adenovirus and several other small DNA tumor viruses disturb host cell growth control by interacting with cellular factors that normally function to repress cell proliferation. The product of the adenovirus E1A gene, is itself sufficient for transformation. E1A transforming activity resides in two distinct domains, the targets of which include p300/CBP and products of the retinoblastoma (RB) susceptibility gene family. Interactions of E1A with p300/CBP and RB are thought to influence functionally distinct growth regulatory pathways, allowing the two domains to contribute additively to transformation.

Although p300/CBP by itself does not bind to DNA, each can be recruited to promoter elements via interaction with sequence-specific activators including CREB, c-Jun, YY1, Fos, c-Myb and nuclear receptors. E1A inhibits the p300/CBP-mediated transcriptional activation of many promoters. Importantly, E1A does not appear to disrupt p300/CBP-activator interactions. This strongly suggests the presence of a cellular counterpart of E1A which binds to p300/CBP and plays an important role in both transcription and cell cycle regulation.

Major Findings

Cloning of P/CAF

We cloned the p300/CBP-associated factor (P/CAF) based on an analogy between species. In yeast, GCN4 is believed to be a c-Jun counterpart on the basis of similarities in DNA recognition as well as the participation of both proteins in UV signaling pathways. Yeast genetic screening has led to the isolation of various cofactors for GCN4, including GCN5, ADA2 and ADA3. Thus, we considered that there might exist human counterparts to GCN5, ADA2, or ADA3 that interact with p300/CBP to mediate transcriptional activation by c-Jun.

We found two human cDNAs encoding GCN5-related polypeptides (ETS05039 and NIB2000-5R) from GenBank. The former encodes an N-terminal region with no obvious sequence similarity to any proteins in the databases besides the GCN5-related C-terminal region, whereas the latter encodes only the GCN5-related region. Given that obvious p300/CBP-binding activity was observed in the former polypeptide as shown below, we named it p300/CBP-associated factor (P/CAF).

E1A disturbs P/CAF-p300/CBP interaction

p300/CBP sites important for P/CAF interaction were determined. In vitro interaction study with various p300/CBP mutants lead us to conclude that P/CAF and E1A bind to the same or very closely spaced sites on p300/CBP. This raises the possibility of direct competition between P/CAF and E1A for binding to p300/CBP. As expected, the interaction of P/CAF with the p300/CBP was progressively inhibited by the addition of increasing amounts of E1A.

In vivo interaction between P/CAF and p300/CBP was demonstrated by coimmunoprecipitation from cell extracts. Importantly, the endogenous interaction of P/CAF with p300 was drastically inhibited by expression of E1A.

Cell cycle regulation by P/CAF (in collaboration with Bruce H. Howard)
Given that binding of P/CAF to p300/CBP is inhibited by E1A, we considered that P/CAF might compete with E1A in its effect on the cell cycle. This possibility was addressed by examining whether transient expression of P/CAF would affect the rate of G1/S transit in HeLa cells. P/CAF negatively affected the distribution of cells between G1 and S phases in this assay. E1A alone has mitogenic activity in this experimental setting. Intriguingly, when P/CAF was cotransfected with E1A, the mitogenic activity of E1A was significantly counteracted by P/CAF. These results support the view that P/CAF and E1A mediate antagonistic effects on cell cycle progression.

Histone acetylase activity of P/CAF
Acetylation of the N-terminal histone tails has been considered to play a crucial role in accessibility of transcription factors to nucleosomal templates. Based on the information that yGCN5 is a histone acetylase, intrinsic histone acetyltransferase activity in P/CAF was examined. P/CAF acetylated histones H3 and H4, but preferentially H3 in both free histones and the mononucleosome.

Implications

Direct function of P/CAF is likely to involve its intrinsic histone acetyltransferase activity. Although exact molecular mechanisms by which acetylation of core histones contribute to transcription remains undefined, acetylation of the histones is considered to play an important role in transcriptional regulation. Our findings provide insights into the mechanisms of targeted histone acetylation (Fig. 2). P/CAF could be recruited onto the specific promoters through selective interactions, then presumably acetylate histones in a promoter-specific manner. Intriguingly, the SWI/SNF complex, known to be a global gene regulator whose functions include remodeling chromatin, functionally interacts with GCN5, ADA2, and ADA3. Thus, targeted histone acetylation could contribute to promoter activation by disrupting the repressive chromatin structure, perhaps in conjunction with the SWI/SNF complex.

p300/CBP binds to various sequence-specific factors that are involved in cell growth and/or differentiation, including CREB, c-Jun, c-Fos, c-Myb and nuclear receptors. P/CAF could stimulate the activation function of these factors via promoter-specific histone acetylation. Our work indicates that E1A perturbs normal cellular regulation by disrupting the connection between p300/CBP and its associated histone acetyltransferase.

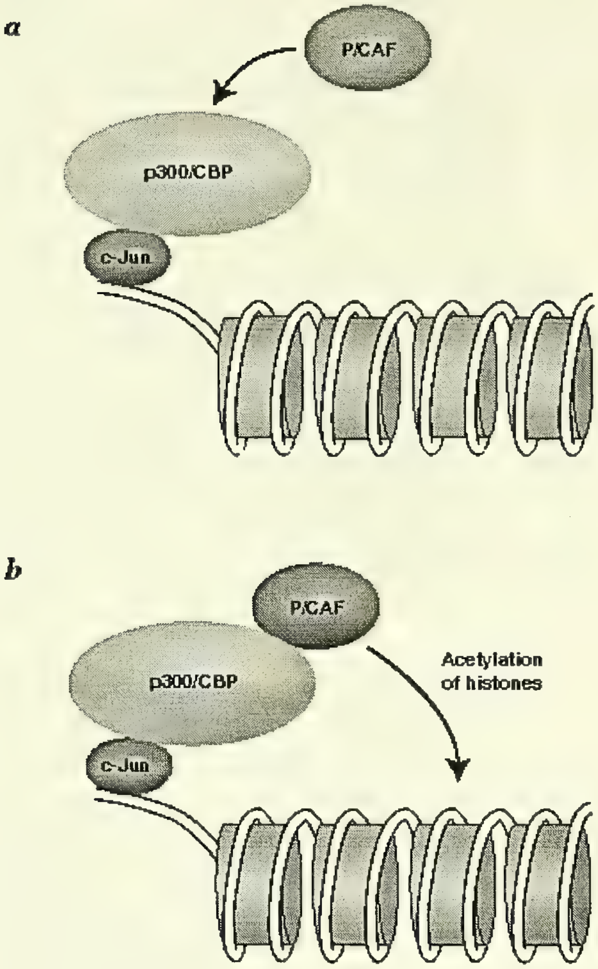


FIG. 2. Model of molecular functions of P/CAF and E1A. p300/CBP could be recruited to promoter elements via interaction with various sequence-specific activators. P/CAF could also be recruited onto the specific promoters through interaction with p300/CBP (*a*), then acetylate histones in a promoter-specific manner (*b*). The E1A oncoprotein perturbs normal cellular regulation between P/CAF and p300/CBP by competing for the binding sites in p300/CBP.

Proposed Course

Histones are acetylated in a promoter-specific manner in vivo. Our findings suggest that recruitment of a histone acetylase on the specific promoters through protein-protein interactions is one of the mechanisms of targeted histone acetylation. In collaboration with Alan Wolffe's laboratory, we plan to demonstrate whether recruitment of the enzyme onto DNA stimulates acetylation, we will express P/CAF as a fusion protein with the GAL4-DNA binding domain. We will use this fusion protein for acetylation of in vitro reconstituted nucleosomal DNA with and without GAL4-binding sites. These studies will provide information as to whether recruitment of an acetylase onto DNA contributes to targeted histone acetylation.

Although acetylation of the histones is considered to contribute to transcriptional activation, its mechanisms are unclear. In collaboration with Bruce Howard's laboratory, we plan to investigate how overexpression of P/CAF affects transcription in cultured cells. As a control, we are attempting to manufacture a P/CAF mutant lacking histone acetylase activity. To understand the functions of P/CAF at the organism level, we started targeting the mouse P/CAF gene in collaboration with Heiner Westphal's laboratory. Targeted mice are also useful to establish P/CAF-knockout cell lines. Since the endogenous level of P/CAF is a potential problem to investigate P/CAF's functions in cultured cells, P/CAF-knockout cells would be useful. In collaboration with Danny Reinberg's laboratory, we have found that both p300 and P/CAF are components of the RNA polymerase II holoenzyme. This indicates that the RNA polymerase II holoenzyme can connect from activators to RNA polymerase II. Moreover, the RNA polymerase II holoenzyme contains the SWI/SNF complex. We plan to reconstitute transcription from nucleosomal templates using the RNA polymerase II holoenzyme and other purified transcription factors.

Publications

Godde JS, Nakatani Y, Wolffe AP. The amino-terminal tails of the core histones and the translational position of the TATA box determine TBP/TFIIA association with nucleosomal DNA, *Nucleic Acids Res.* 1995;**23**:4557-64

Hisatake K, Ohta T, Takada R, Guermah M, Horikoshi M, Nakatani Y, Roeder RG. Evolutionary conservation of human TATA-binding-polypeptide associated factors TAFII31 and TAFII80 and interactions of TAFII80 with other TAFs and with general transcription factors. *Proc. Natl. Acad. Sci. U. S. A.* 1995;**92**:8195-9.

Nakatani Y, Bagby S, Ikura M. The histone folds in transcription factor TFIID *J. Biol. Chem.* 1996;**271**:6575-8.

Xie X, Kokubo T, Cohen SL, Mirza UA, Hoffmann A, Chait BT, Roeder RG, Nakatani Y, Burley SK. Structural similarity between TAFs and the heterotetrameric core of the histone octamer. *Nature* 1996;**380**:316-22.

Hoffmann A, Chiang C-M, Oelgeschläger T, Xie X, Burley SK, Nakatani Y, Roeder RG. A histone octamer-like structure within TFIID. *Nature* 1996;**380**:356-9.

Yang X-J, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y.
A p300/CBP-associated factor that competes with the adenoviral E1A oncoprotein. *Nature* 1996;**382**:319-24.

Farmer G, Colgan J, Nakatani Y, Manley JL, Prives C. A functional interaction between p53, TBP and TAFs in vivo. *Mol. Cell. Biol.* in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD00506-01 LMGR

PERIOD COVERED

January 7, 1996 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Initiation of DNA Replication in Mammalian Chromosomes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Melvin L. DePamphilis	Section Chief	LMGR, NICHD
Others:	Bender, Thomas	Summer Student	LMGR, NICHD
	Kobayashi, Takehiko	Visiting Fellow	LMGR, NICHD
	Li, Cong-Jun (started 9/96)	Post-Doctoral Fellow	LMGR, NICHD
	Natale, Darren A.	IRTA	LMGR, NICHD
	Rein, Theo	Guest Worker	LMGR, NICHD
	Gilbert, David M.	Research Associate	Roche Institute

COOPERATING UNITS *(if any)*

See attached

LAB/BRANCH

Laboratory of Molecular Growth Regulation

SECTION

Section on Eukaryotic DNA Replication

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.6	4.5	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The key step in regulating the proliferation of cells and viruses is control of initiation of DNA replication. Central to understanding how this control works is understanding the nature of sites where replication begins (i.e. origins of DNA replication). We and others have developed various strategies for mapping the locations of initiation sites in the chromosomes of cells from metazoan animals to a resolution of ≤1 kilobase (kb). At least 18 specific initiation sites have now been mapped in mammalian chromosomes. In general, most initiation events at these sites occur within a 0.5 to 2 kb "origin of bidirectional replication" (OBR) although initiation events can be detected throughout a larger "initiation zone" of up to 60 kb. We have shown that site specific initiation of DNA replication results from the interaction between specific DNA sequences and nuclear (chromatin?) structure. Moreover, others have shown that specific initiation sites are both developmentally acquired and established during the mid to late G1-phase of their cell division cycle. We have developed an assay for cis-acting sequences required for site specific initiation by transfecting hamster cells with plasmids containing a well characterized OBR (ori-β). These ectopic ori-β sequences behave like active replication origins, while ectopic copies of sequences from the replication initiation inactive regions remain inactive. Therefore, origin activity depends on specific DNA sequences. Analysis of DNA methylation at two replication origins (ori-β, ori-RPS14) revealed that both OBRs are associated with a high density cluster of methylated CpG dinucleotides throughout their cell division cycle. However, neither OBR contained the putative densely methylated island (DMI) reported by Tasheva and Roufa [Mol. Cell. Biol. 14: 5636-44, 1994] in which all cytosines are methylated in proliferating cells. Remethylation studies demonstrated directly that active OBR sequences contained the same methylation pattern as bulk OBR sequences. Other mammalian OBRs have the potential for similar methylation patterns, suggesting that these ^{m}CpG clusters may play a role in either the establishment or regulation of mammalian replication origins. We are now attempting to identify specific sequences that contribute to site specific initiation, and the proteins that interact with these sequences.

Cooperating Units:

The PI began his appointment at the NIH on January 7, 1996. During 1995, this research was carried out at the Roche Institute of Molecular Biology, Nutley, NJ 07110.

Haralabos Zorbas, Dept. Biochemistry, Ludwig Maximilians-Universität, Würmtalstrasse 221, D-81375 München, Germany.

John Diffley, ICRF, Clare Hall Laboratories, South Mimms, Herts. EN6 3LD, England

Project Description

Objective

To understand how mammalian cells decide when and where to initiate DNA replication in their chromosomes.

Major Findings

1. Mimosine Arrests DNA Synthesis at Replication Forks by Inhibiting Deoxyribonucleotide Metabolism

Mimosine has been reported to specifically prevent initiation of DNA replication in the chromosomes of mammalian nuclei. In order to test this hypothesis, the effects of mimosine were examined in several different DNA replication systems and compared with the effects of aphidicolin, a specific inhibitor of replicative DNA polymerases. Our results demonstrate that, while DNA replication in mammalian nuclei and mitochondria share little in common, mimosine inhibited DNA synthesis in both genomes to the same extent. Similarly, DNA replication in simian virus 40 (SV40) and cell chromosomes utilize the same proteins and replication fork mechanism to synthesize DNA, but differ in their initiation process. Nevertheless, mimosine inhibited replication in both genomes to the same extent. Furthermore, mimosine and aphidicolin were indistinguishable in their ability to arrest replication forks in SV40 DNA replicating intermediates, and to arrest replication forks in mammalian nuclear chromosomes close to sites of known origins. However, in contrast to aphidicolin, mimosine did not inhibit DNA replication in lysates of mammalian cells. Mimosine also had no effect on initiation or elongation of DNA replication in *Xenopus* eggs or egg extracts. These data suggested that mimosine inhibits some process common to DNA synthesis rather than initiation of DNA replication, such as deoxyribonucleotide metabolism. In fact, mimosine decreased intracellular levels of dATP in parallel with its inhibitory effect on DNA synthesis, and dATP levels and DNA synthesis were restored by adding iron to mimosine treated cells. Taken together, these results show that mimosine inhibits DNA synthesis by reducing deoxyribonucleotide pools via an inhibitory effect on a key enzyme involved in deoxyribonucleotide metabolism, ribonucleotide reductase, and that mimosine does not specifically inhibit initiation of DNA replication.

2. Site-Specific Initiation of DNA Replication in *Xenopus* Egg Extract Requires Nuclear Structure

Previous studies have shown that *Xenopus* egg extract can initiate DNA replication in purified DNA molecules once the DNA is organized into a pseudo-nucleus. DNA replication under these conditions is independent of DNA sequence and begins at many sites distributed randomly throughout the molecules. In contrast, DNA replication in the chromosomes of cultured animal cells initiates at specific, heritable sites. Here we show that *Xenopus* egg extract can initiate DNA replication at specific sites in mammalian chromosomes, but only when the DNA is presented in the form of an intact nucleus. Initiation of DNA synthesis in nuclei isolated from G1-phase Chinese Hamster Ovary (CHO) cells was distinguished from continuation of DNA synthesis at preformed replication forks in S-phase nuclei by a delay that preceded DNA synthesis, a dependence on soluble *Xenopus* egg factors, sensitivity to a protein kinase inhibitor, and complete labeling of nascent DNA chains. Initiation sites for DNA replication were mapped downstream of the amplified dihydrofolate reductase (DHFR) gene region by hybridizing newly replicated DNA to unique probes and by hybridizing Okazaki fragments to the two individual strands of unique probes. When G1-phase nuclei were prepared by methods that preserved the integrity of the nuclear membrane, *Xenopus* egg extract initiated replication specifically at or near the origin of bidirectional replication utilized by hamster cells (DHFR ori–β). However, when nuclei were prepared by methods that altered nuclear morphology and damaged the nuclear membrane, preference for initiation at ori-β was significantly reduced or eliminated. Furthermore, site specific initiation was not observed with bare DNA substrates, and *Xenopus* eggs or egg extracts replicated prokaryotic DNA or hamster DNA that did not contain a replication origin as efficiently as hamster DNA containing ori–β. We conclude that initiation sites for DNA replication in mammalian cells are established prior to S-phase by some component of nuclear structure, and that these sites can be activated by soluble factors in *Xenopus* eggs.

3. Absence Of An Unusual "Densely Methylated Island" At The Hamster DHFR Ori–β

An unusual "densely methylated island" (DMI), in which all cytosine residues are methylated on both strands for one to five hundred base pairs, has been reported at mammalian origins of DNA replication. Since this DMI appeared in about 90% of proliferating cells, but not in stationary cells, it may regulate origin activation. In an effort to confirm and extend these observations, the DMI at the well characterized ori–β locus 17 kb downstream of the DHFR gene in chromosomes of Chinese hamster ovary cells was examined using three independent methods for detecting methylated cytosines in genomic DNA. The methylation status of this region was examined in randomly proliferating and stationary cells, and in cell populations enriched in the G_1, S or G_2+M phases of their cell division cycle. DNA was subjected to (1) cleavage by a methylation sensitive restriction endonuclease, (2) hydrazine modification of cytosines followed by piperidine cleavage, and (3) permanganate modification of 5-methylcytosines (^{m}C) followed by piperidine cleavage. The permanganate reaction is a novel method for direct detection of ^{m}C residues that complements the more commonly used hydrazine method. Products of these reactions were identified using either direct blotting and hybridization or the ligation mediated polymerase chain reaction. These methods were capable of detecting ^{m}C in 6 to 10% of the cells. Nevertheless, the ori–β DMI was not detected in any of these cell populations using any of these methods.

4. DNA Methylation at Mammalian Replication Origins

The *E. coli* replication origin contains numerous DNA methylation sites that play a role in regulating initiation of DNA replication. To ascertain whether DNA methylation might also play a role in mammalian replication origins, we examined the well-characterized origin of bidirectional replication (OBR) located 17 kb downstream of the DHFR gene in Chinese hamster ovary cell chromosomes (ori–β). The two kb region of ori–β that exhibits greatest replication initiation activity also contains numerous potential methylation sites in the form of CpG dinucleotides. Using bisulfite to chemically distinguish cytosine (C) from 5-methylcytosine (^{m}C), all twelve CpGs within the two kb region were found to be methylated, nine of which were located within 356 bp flanking the minimal OBR. The density of methylated sites was about 10 times greater than the average for mammalian DNA, consistent with a role for methylation at replication origins. However, the previously reported densely methylated island (DMI), in which all cytosines within a 516 bp region were methylated, was not detected using either bisulfite or methylation-sensitive restriction endonucleases. These results are in full agreement with recent findings using alternative methods for detecting ^{m}C. Similar results were obtained at the RPS14 OBR.

Remethylation studies demonstrated directly that active origins contained the same methylation pattern as bulk origins. Moreover, the organization of CpG dinucleotides at other mammalian origins revealed the potential for similar methylation patterns, suggesting that these high density methylation clusters may play a role in either the establishment or regulation of mammalian replication origins.

5. Genetic Analysis Of Mammalian Replication Origins

Initiation of DNA replication during S-phase in cultured mammalian cells does not occur randomly throughout chromosomes, but at specific DNA sites. However, these initiation sites appear more complex than would have been anticipated by analyses of simpler genomes. For example, at ori-β located about 17 kb downstream from the DHFR gene in hamster cells, application of several mapping methods in which nascent DNA strands are radiolabeled or density labeled concluded that most initiation events occurred within an 0.5 to 2 kb locus [origin of bidirectional replication (OBR)].

However, application of the neutral/neutral 2-D gel electrophoresis mapping method to identify replication bubble structures concluded that initiation events occurred randomly throughout a 55 kb region that included the OBR. In an effort to resolve this paradox, the method of Giacca et al. [PNAS **91**: 7119 (1994)] was used to measure the relative abundance of newly synthesized DNA throughout this genomic region when CHO cells entered S-phase. The results demonstrated that >90% of initiation events occurred at the OBR. To determine whether or not the OBR locus was a cis-dominant genetic element, DNA fragments containing ori–β were transferred to other chromosomal sites at random, and assayed for their ability to initiate replication using the Giacca method. In addition, sequences from the DHFR gene where initiation events have never been detected by any method and sequences from λ–DNA that should not contain a eukaryotic origin were also tested. Most of these ectopic ori–β sequences functioned as replication origins, while most of the non-origin sequences did not. These results demonstrated that origin activity in mammalian chromosomes depends on specific DNA sequences.

Proposed Course of Project

Current research is directed at four specific goals: (1) Identification of specific DNA sequences that promoter initiation of DNA replication in mammalian chromosomes. (2) Identification of novel features in the DNA (e.g. methylation sites) or chromatin (e.g. hypersensitive sites, footprints, histone hyperacetylation) at replication origins, and determine whether or not these features are important to origin activity. (2) Cloning and characterization of the six mammalian homologues of the yeast origin recognition complex (ORC). This protein complex binds specifically to yeast replication origins, and we anticipate that the mammalian homologues will do the same. (3) Map the locations of mammalian ORC in mammalian chromosomes and determine whether or not it occupies a specific site such as ori–β in the CHO cell genome.

Significance to Biomedical Research and the Program of the Institute

Our institute is dedicated to improving child health and human development. What could be more germane to this task than understanding how mammalian cells regulate replication of their genome. DNA replication is the primary event that regulates cellular and viral proliferation. Agents that block DNA replication can arrest the spread of cancer cells and eliminate viral pathogens. Gene amplification, on the other hand, is one of the primary mechanisms by which cancer cells become resistant to drug therapy. Thus, understanding how cells regulate initiation of DNA replication is essential to understanding how cells proliferate, to designing rational drug therapies, and to constructing an "artificial chromosome" that replicates in mammalian cells. Artificial chromosomes would allow new genetic information to be introduced into cells and animals without interference of its regulation by cellular DNA sequences.

Publications

DePamphilis, M.L. (1995) Specific Labeling of Newly Replicated DNA, pp. 628-669 *In* Methods in Enzymology - DNA Replication, vol. 262 (J. Campbell, ed.), Academic Press, NY.

DePamphilis, M.L. (1996) Origins of DNA Replication, pp. 45-86 *In* DNA Replication in Eukaryotic Cells, (M.L. DePamphilis, ed) Cold Spring Harbor Laboratory Press, NY.

DePamphilis, M.L. (1996) Replication Origins in Metazoan Chromosomes, pp. in Eukaryotic DNA Replication - Frontiers in Molecular Biology (J. Blow, ed), Oxford University Press, England.

DePamphilis, M.L. (ed), DNA Replication in Eukaryotic Cells, Cold Spring Harbor Laboratory Press, NY, 1996.

Gilbert, D.M., H. Miyazawa, and M. L. DePamphilis. (1995) Site-Specific Initiation of Mammalian DNA Replication in *Xenopus* Egg Extract Requires Nuclear Structure, Mol. Cell. Biol. **15**: 2942-2954.

Gilbert, D.M., Neilson, A., Miyazawa, H., DePamphilis, M.L. and Burhans, W.C. (1995) Mimosine Arrests DNA Synthesis at Replication Forks by Inhibiting Deoxyribonucleotide Metabolism, J. Biol. Chem. **270**: 9597-9606.

Kobayashi, T. and M. L. DePamphilis. (1996) Specific Sequences Promote Site Specific Initiation of DNA Replication in Mammalian Chromosomes, in preparation.

Rein, T., D.A. Natale, H. Zorbas, and M.L. DePamphilis. (1996) DNA Methylation At Mammalian Replication Origins, submitted.

Rein, T., T. Kobayashi, and M. L. DePamphilis. (1996) Remethylation of DNA at a Specific Mammalian Origin of Bidirectional Replication , in preparation.

Rein, T., U. Gärtner, M. Niggemann, M. L. DePamphilis, and H. Zorbas. (1996) Absence Of An Unusual "Densely Methylated Island" At The Hamster DHFR Ori–β, submitted.

Salas, M., J.T. Miller, J. Leis and M.L. DePamphilis. (1996) Mechanisms for Priming DNA Synthesis, pp. 131-176 *In* DNA Replication in Eukaryotic Cells, (M.L. DePamphilis, ed) Cold Spring Harbor Laboratory Press, NY.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD00507-01 LMGR

PERIOD COVERED

January 7, 1996 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)* Regulation of DNA Replication and Gene Expression at the Beginning of Mammalian Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Melvin L. DePamphilis	Section Chief	LMGR:NICHD
Others:	Kaneko, Kotaro	Visiting Associate	LMGR:NICHD
	Miranda, Miriam	Research Assistant	Roche Institute
	Nothias, Jean-Yves	Research Associate	Roche Institute
	Wiekowski, Maria	Research Associate	Roche Institute

COOPERATING UNITS *(if any)*

See attached

LAB/BRANCH

Laboratory of Molecular Growth Regulation

SECTION

Section on Eukaryotic DNA Replication

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Life begins when a single egg is fertilized by a single sperm to set in motion a sequence of events that include remodeling and replicating each of the parental genomes before combining them into a single nucleus and expressing a tiny fraction of their genes. We have developed a novel approach to identifying cis-acting sequences and trans-acting factors that are required to activate DNA replication and gene expression at the beginning of mammalian development. Plasmid DNA encoding a sensitive reporter gene linked to eukaryotic regulatory sequences is injected into the nuclei of mouse oocytes, fertilized eggs (1-cell embryos) and 2-cell embryos (where zygotic gene activation occurs). The results of such studies have revealed that replication and expression of genes encoded in the injected, extrachromosomal DNA responded to the same signals that regulate these functions in cellular DNA: They required specific cis-acting regulatory sequences and the trans-acting proteins that activate them, and they occurred only when the host cell executed the same function on its own genome. These results, taken together with analyses of endogenous gene expression and results from nuclear transplantation studies, revealed several novel features of zygotic gene expression at the beginning of mammalian development. A time dependent mechanism ("zygotic clock") delays transcription of zygotic genes and translation of nascent mRNA until the 2-cell stage in development. This provides an opportunity to remodel chromatin structure of parental genomes and possibly facilitate genomic imprinting without accidentally exposing genes to premature expression. In addition, the ability to use enhancers to activate promoters or replication origins does not appear until formation of 2-cell embryo, because an enhancer specific co-activator is absent prior to this stage in development. Therefore, the first two S-phases in mouse development occur under conditions where enhancers cannot function, a fact that may contribute to developmental acquisition of site specific initiation of DNA replication. The need for a TATA-box to facilitate promoter activity appears only after cell differentiation occurs and then only as a mediator of enhancer function. Prior to activation of zygotic genes in 2-cell embryos, the activity of promoters and replication origins is repressed by changes in chromatin structure, allowing positive regulatory factors to activate genes selectively. One of the first transcription factors to be expressed at the beginning of mouse development is TEAD2, a member of a new gene family expressed throughout animal development. TEAD2 is expressed specifically during formation of a 2-cell embryo and is able to stimulate strongly the activity of appropriate enhancers, presumably turning on the next cascade of genes in the developmental program.

Cooperating Units

The PI began his appointment at the NIH on January 7, 1996. During 1995, this research was carried out at the Roche Institute of Molecular Biology, Nutley, NJ 07110.

Sadhan Majumder, Dept. Molecular Genetics, Univ. Texas MD Anderson Cancer Center, Houston, TX 77030, USA

PROJECT DESCRIPTION

Objective

To understand how the processes of DNA replication and transcription are turned on at the beginning of mammalian development.

Major Findings

1. Repression of Gene Expression At the Beginning of Mouse Development

The transition from maternal to zygotic gene expression in the mouse occurs in the 2-cell embryo. Previous studies in which DNA was injected into 2-cell embryos revealed that transcription promoters and origins of DNA replication are strongly repressed in cleavage stage embryos unless linked to an embryo-responsive enhancer. Repression also occurs when DNA is injected into the paternal pronucleus of a 1-cell embryo and the injected embryo subsequently undergoes mitosis, except that repression is no longer relieved by enhancers. Here we extend this observation to maternal pronuclei in 1-cell embryos, and show that this repression could not be relieved either by linking the promoter to an embryo-responsive enhancer or by inducing hyperacetylation of chromatin in order to increase its accessibility to transcription factors. However, repression could be relieved by transplanting the injected pronucleus to a 2-cell embryo, even when the recipient cell subsequently underwent mitosis. Both the extent of promoter repression and the ability of enhancers to stimulate promoter activity increased as development proceeded from the early 2-cell stage to the 4-cell stage. Once repression was established in late 2-cell embryos, transplanting an injected 2-cell embryo nucleus back to an early 1-cell embryo failed to restore activity to the injected promoter, even when it was linked to an enhancer. These and other data demonstrate that cytoplasmic factors appear during formation of a 2-cell embryo that can repress promoter activity and activate enhancer activity. These factors are absent from the paternal pronucleus and cytoplasm of early (S-phase arrested) 1-cell embryos. Moreover, the cytoplasm of early 1-cell embryos appears to lack the ability to reprogram expression of genes once they have progressed to the late 2-cell stage in mouse development.

2. Uncoupling of Transcription and Translation During Zygotic Gene Activation in the Mouse

Zygotic gene expression in mice is delayed by a time dependent mechanism until the 2-cell stage in development. To investigate the basis of this "zygotic clock", the firefly luciferase gene was injected into mouse embryos, and quantitative assays were used to monitor luciferase gene transcription and translation in individual embryos from single mothers. These studies confirmed, at the mRNA level, previous conclusions about the relative capacities of paternal and maternal pronuclei to transcribe genes and the requirements for promoters and enhancers during zygotic gene activation. Furthermore, these studies revealed that fertilized mouse eggs can delay expression of zygotic genes by uncoupling translation from transcription. An RNA polymerase II dependent gene could be transcribed prior to cell cleavage, but it was not translated until zygotic gene expression began (a delay of up to 15 hours after injection). The time course for nascent mRNA accumulation was biphasic, with the second phase occurring during zygotic gene expression. If the luciferase gene was injected after zygotic gene expression had begun, then translation was tightly linked to transcription. If the second phase of mRNA accumulation was repressed, then luciferase was not produced. Therefore, translation was linked to the accumulation of mRNA during the onset of zygotic gene expression. Similar biphasic time courses also were observed for RNA polymerase I and III dependent transcription. These and other results reveal that the zygotic clock regulates the onset of both transcription and translation of zygotic genes.

3. Changes in Histone Synthesis And Modification at the Beginning of Mouse Development Correlate With the Establishment of Chromatin Mediated Repression of Transcription

Previous studies have shown that the transition from a late 1-cell mouse embryo to a 4-cell embryo, the period when zygotic gene expression begins, is accompanied by an increasing ability to repress the activities of promoters and replication origins. Since this repression can be relieved by either butyrate or enhancers, it appears to be mediated through chromatin structure. Here we identify changes in the synthesis and modification of chromatin bound histones that are consistent with this hypothesis. Histone synthesis up to the early 2-cell stage was insensitive to α–amanitin, and therefore originated from maternal mRNA inherited from the oocyte. Histones H3 and H4 were synthesized in early 1-cell embryos, but synthesis of histones H2A, H2B and H1 (proteins required for chromatin condensation) was delayed until the late 1-cell stage. Moreover, histone H4 in both 1-cell and 2-cell embryos was predominantly diacetylated (a modification that facilitates transcription), while H4 in oocytes and fibroblasts was predominantly unacetylated or monoacetylated. This reduction in acetylation began during the late 2-cell to 4-cell stage. Arresting development at the beginning of S-phase in 1-cell embryos suppressed the ability of these cells both to repress transcription and to synthesize histones. Thus, the onset of chromatin-mediated repression at the beginning of mouse development was consistent with changes in the production of histone H1 and acetylation of core histones.

4. A Novel Gene Family that Binds to the TEF-1 Transcription Factor DNA Binding Site Is Expressed at the Beginning of Zygotic Gene Expression in the Mouse

A survey of polyomavirus mutants that replicate in undifferentiated mouse embryonal carcinoma or embryonic stem cells (derived from the inner cell mass that gives rise to the embryo itself) identified the F101 polyomavirus enhancer as the most effective in stimulating the activity of promoters injected into 2-cell mouse embryos. Stimulation ranged from 20 to >300-fold, and its activity depended on two tandem DNA binding sites for transcription factor TEF-1 with an optimal center-to-center spacing of ~50 bp (Martínez-Salas et al., Genes & Devel. **3**: 1493-1506, 1989; Mélin et al., EMBO J. **12**: 4657-4666, 1993). TEF-1 is a ubiquitously expressed, highly conserved transcription factor that has been implicated in mediating cell-specific transcription in skeletal and cardiac muscle cells, as well as in keratinocytes, neural precursor cells, and syncytiotrophoblasts of the placenta (Blatt and DePamphilis, Nucl. Acids Res. **21**: 747-748, 1993). However, since TEF-1 itself is not required for preimplantation development (Chen et al., *Genes Devel* **8**, 2293-2301, 1994), another member of this family may activate enhancers in preimplantation embryos. We have identified two additional, novel genes whose putative DNA binding domains are 100% homologous at the amino acid level to that of TEF-1. Analysis of restriction endonuclease fragments suggests strongly that mammals contain only three such genes which we refer to as TEA-domain (TEAD) gene family, although they appear to contain additional family members that do not share a perfect TEA domain homology. As expected, TEAD1 (TEF-1), TEAD2 and TEAD3 all bind efficiently to the TEF-1 DNA binding element *in vitro*. Moreover, all three transcripts are ubiquitously expressed in most adult tissues, although the relative abundance of these transcripts can differ significantly among tissues. Which of these genes is responsible for activating the polyomavirus F101 enhancer at the beginning of mouse development?

Neither TEAD1, 2 or 3 mRNA was detected in oocytes using *in situ* hybridization, although TEAD2 was detected in embryos by day 7 and in the follicle cells surrounding the oocyte, suggesting that TEAD2 was expressed specifically sometime during preimplantation development. However, RT-PCR detected all three mRNAs in oocytes and eggs, although TEAD1 and TEAD3 mRNAs disappeared rapidly after formation of a 2-cell embryo while TEAD2 mRNA accumulated as development proceeded to the blastocyst stage. Therefore, all three genes appeared to be expressed at low levels in oocytes, but only TEAD2 was expressed in the zygote. A quantitative application of RT-PCR (Rambhatla et al., Mol. Reprod. Dev. 41, 314-324, 1995) revealed that TEAD2 mRNA is expressed at 4000 - 6000 copies per ovum from oocytes up to the early 2-cell stage, at which time, the levels decline and then rise again reaching approximately 100,000 copies per embryo by the blastocyst stage. This high level of expression at the blastocyst stage is comparable to that of actin. TEAD3 mRNA was not detected in preimplantation embryos, and TEAD1 is currently under investigation. The facts that only TEAD2 is expressed strongly throughout preimplantation development, and that the TEAD1/TEF-1 gene is not required until day 12 suggest that the ability of early preimplantation embryos to utilize polyomavirus F101 enhancer results from expression of TEAD2 when zygotic gene expression begins. Injection of a TEAD-dependent promoter linked to a reporter gene revealed that functional TEAD protein does not appear until the late 1-cell stage, suggesting that translation of TEAD mRNAs present in oocytes is delayed. This was similar to expression of mouse histone H1 gene where maternally inherited mRNA was not translated until the late 1-cell stage. Interestingly, the TEAD2 gene, whose maternal message persists until the late 2-cell and early 4-cell embryos, contains a sequence homologous the Cytoplasmic Polyadenylation Element (CPE) located in the 3'-untranslated region where CPEs have been found previously for other developmentally regulated maternal messages.

Thus, it appears that TEAD2 is one of the first transcription factors expressed from the newly formed zygotic genome during preimplantation development. Presumably, TEAD2 protein triggers transcription of specific embryonic genes in preimplantation mouse embryos that are linked to an appropriate TEAD responsive enhancer. TEAD2 also plays an important role in development of neural tissue at later stages in mouse development (Yasunami et al., J. Biol. Chem. 270, 18649-54, 1995). *In situ* hybridization studies indicate that the TEAD genes are differentially expressed in developing decidua (in the uterus) as well as in developing follicles (in the ovary), suggesting their importance in growth and differentiation of cells throughout mammalian development.

5. Developmental Acquisition of Enhancer Function Requires A Co-Activator Activity

Enhancer function first appears during mouse development upon formation of a 2-cell embryo, concurrent with the major onset of zygotic gene expression. Injection of a plasmid encoded reporter gene into the nuclei of oocytes, 1-cell and 2-cell embryos revealed that enhancers could not stimulate promoter activity, even in the presence of a sequence-specific activation protein. GAL4:VP16 efficiently activated a GAL4-dependent promoter in these cells, but it did not activate a GAL4-dependent enhancer until formation of a 2-cell embryo. Although chromatin mediated repression was absent in the paternal pronucleus of 1-cell embryos, it was present in the maternal nuclei of both oocytes and 1-cell embryos and in the zygotic nuclei of 2-cell and 4-cell embryos. Therefore, the inability to elicit enhancer function prior to formation of a 2-cell embryo did not result from the absence of either promoter repression or sequence specific enhancer activation proteins. Instead, enhancer function required a specific co-activator activity that was detected only after formation of a 2-cell embryo. Enhancer function could be suppressed in 2-cell embryos by competition with unrelated enhancers, but not with promoters. Competition was not detected in 1-cell embryos. Moreover, enhancer function could be partially restored in oocytes by preinjection of mRNA from embryonic stem cells. Enhancer function in 2-cell and 4-cell embryos was unaffected by this procedure. Therefore, enhancer function *in vivo* requires the presence of a unique co-activator activity in addition to chromatin mediated repression and enhancer specific DNA binding proteins. The fact that this co-activator does not appear during mouse development until formation of a 2-cell embryo suggests that developmental acquisition of enhancer function contributes to the delay of zygotic gene expression that generally occurs during animal development.

Proposed Course of Project

The future of this project depends on the future availability of laboratory space, personnel and resources. Injection experiments on early mammalian development require a full time, highly skilled technician, and a dedicated laboratory for this purpose. At this time, we have stopped all injection experiments. Therefore, Dr. Kaneko alone and through collaborative arrangements with other laboratories will continue these studies in three directions. First, he will attempt to identify the putative enhancer specific co-factor using the one-hybrid system in yeast to detect a mammalian factor that can mediate long range interactions. Second, he will attempt to clone the TEAD2 and TEAD3 genes in an effort to identify the upstream sequences that are used to initiate transcription of one of the first zygotic genes (TEAD2) to be expressed in the mouse. The human TEAD1 gene has already been cloned. Third, he will employ antisense technology to block expression of these genes at the beginning of mouse development, and differential display to attempt to identify genes that are activated by TEAD2 in preimplantation mouse embryos.

Significance to Biomedical Research and the Program of the Institute

Our institute is dedicated to improving child health and human development. What could be more germane to this task than understanding how mammalian life begins when an egg is fertilized by a sperm. This single act initiates a developmental program that ultimately produces a unique and complex animal consisting of trillions of cells organized into hundreds of different tissues and cell types. A clear understanding of the initial events in this process can be applied directly to development of *in vitro* fertilization techniques to aid childless couples, contraception techniques to limit population growth in the next century, and bioassay techniques for detection of genetic defects in a single blastomeres taken from a preimplantation embryo.

Publications

Henery, C. C., M. Miranda, M., Wiekowski, I. Wilmut, and M.L. DePamphilis. (1995) Repression of Gene Expression At the Beginning of Mouse Development, Devel. Biol. **169**: 448-460.

Kaneko, K., E. Cullinan and M.L. DePamphilis. (1996) A Novel Gene Family that Binds to the TEF-1 Transcription Factor DNA Binding Site Is Expressed at the Beginning of Zygotic Gene Expression in the Mouse, in preparation.

Majumder, S. and M.L. DePamphilis. (1995) A Unique Role For Enhancers Is Revealed During Early Mouse Development, BioEssays **17**: 879-889.

Majumder, S., Z. Zhao, K. Kaneko and M.L. DePamphilis. (1996) Developmental Acquisition of Enhancer Function Requires A Co-Activator Activity, submitted.

Nothias, J.-Y., S. Majumder, K.J. Kaneko and M.L. DePamphilis. (1995) Regulation of Gene Expression at the Beginning of Mammalian Development, J. Biol. Chem. **270**: 22077-22080.

Nothias, J.-Y., M. Miranda and M.L. DePamphilis. (1996) Uncoupling Of Transcription And Translation During Zygotic Gene Activation In The Mouse, EMBO J., in press.

Wiekowski, M., M. Miranda, B. M. Turner and M. L. DePamphilis. (1996) Repression Of Gene Expression At The Beginning Of Mouse Development Correlates With Changes In Histone Synthesis And Modification, submitted.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD01310-10 LMGR

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Developmental Gene Regulation of the Immune System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: K. Ozato Head, SMGI, LMGR, NICHD

See Attached

COOPERATING UNITS *(if any)*

Laboratory of Immunopathology, NIAID (Dr. Herbert Morse)

LAB/BRANCH

Laboratory of Molecular Growth Regulation

SECTION

Section on Molecular Genetics of Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-2753

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
11.2	9.5	1.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This group works on gene regulation in the immune system. Our focus has been to elucidate the role of two transcription factors, RXR_ and ICSBP. RXR_ is a member of the large nuclear hormone receptor superfamily. It is capable of heterodimerizing with other receptors is involved in ligand dependent transcription. ICSBP is a member of the interferon regulatory factor (IRF) family and plays a role in gene regulation mediated by interferons and viruses.

We have studied what role RXR plays, forming a heterodimer with RAR in retinoid mediated gene regulation. Synthetic retinoids that are specific for RAR and RXR have been tested for transcriptional activity of heterodimers. We found that RAR ligands (but not RXR ligands) stimulate transcription. Although an RXR ligand alone was not active in stimulating transcription when it was added together with an RAR ligand strong synergistic activation was noted. The co-administration of the two ligands led to synergistic enhancement of biological activity in P19 cells, as neuronal differentiation and apoptosis were both dramatically increased. Similarly increased teratogenic effect was seen in frog and zebrafish development by the two ligands. In vivo footprinting analysis showed that the RXR ligand although itself did not induce the receptor heterodimer occupancy at the RARE, a combination with an RAR ligand stabilized occupancy. These and data obtained previously in this laboratory show that RXR is an active partner of the heterodimer, and participate in transcription.

The in vivo function of ICSBP was studied using ICSBP-/- mice. these mice present a striking abnormality in that myeloid cells are markedly increased in bone marrow, spleen and lymph nodes even from an early stage. The myeloid cells in ICSBP-/- mice are mostly GR-1+/MacI+ cells, and are morphologically typical granulocytes. Cytokine gene expression was examined with quantitative RT-PCR, which showed that while IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, TNF and CSFs are expressed normally expression of IL-12 was markedly reduced in ICSBP-/- mice. IL-12 is a "master" cytokine produced in activated macrophages, and is shown to control expression of IFN_ in TH1 cells. As a results of defective IL-12 production ICSBP-/- mice are defective in expressing IFN_, a cytokine with anti-viral and bactericidal activities, that also stimulates T cell mediated immune responses. Taken together our results indicate that ICSBP plays a critical role in host defense.

Principal Investigator
Keiko Ozato, Ph.D

Other Personnel:
Professional

			Starting Year
Anup Dey	Ph.D	Visiting Associate	
Nisan Bhattacharyya	Ph.D	NRC associate	1994
Jorge Blanco	Ph.D	Visiting Fellow	1992
Cristina Contursi	Ph.D	Visiting Fellow	1995
Chun Hong	MD. Ph.D	NRSA Fellow	1994
Jing Lou	MD. Ph.D	NRS associate	1994
Saverio Minucci	MD	Visiting Associate	1992
Kazushige Sasaki	MD	Visiting Fellow	1995
I-Ming Wang	Ph.D	IRTA	1993
Atsuko Masumi	Ph.D	Visiting Fellow	

Other

Yaffa Rubinstein	BS	Pre-IRTA	1995
Farideh Chitsaz	MS	Pre-IRTA	1996
Shawn Kahn	Professional service C		1995
Dennis Yun	Summer IRTA		
Tamara Sacks	Summer IRTA		
Robin Kang	Summer IRTA		

Total Man year 12.2 Pofessional 9.5 Others 1.7

Objectives

The overall ojective of this program is to elucidate the mechanisms of gene regulation relavant to the development of the immune system. Within this framework, we analyze transcription of MHC class I and other genes that are regulated by retinoids and interferons.

We have previously isolated and studied several genes encoding proteins that bind to the regulatory DNA elements of MHC class I and other genes. These genes are regulatory genes that belong to large families, and encode proteins that contain distinct DNA binding domains. We found that these DNA binding proteins bind cis elements of not only MHC class I genes but many other genes. Based on the multiple binding specificities we have proposed a "combinatorial model" for gene regulation. In this model regulation of individual genes is controlled by multiple transcription factors bound to multiple regulatory elements, and the patterns of the combination of factors determine specificity of transcription. This is because transcription factors are not unique to each gene but are shared by many genes. From this model it is expected that the functions of these DNA binding proteins are broad, and pleiotropic. The main objective of our research is to study their regulatory roles.

To analyze the mechanisms of transcription, it is important to establish a model system in which each and every component involved in transcription can be identified and dissected. An in vitro transcription assay using the G-free cassette is suitable for this purpose. Our current goal is to apply this assay for studying mechanisms of the action of cloned DNA binding proteins.

In the living cells chromosomal genes interact with nucleosomes and chromatin and are organized into higher orders. In the intact nucleus, cis regulatory elements may be exposed to a special microenvironment, where some transcription factors are locally concentrated or depleted. Such microenvironments may be far from the environment in nuclear extracts prepared in a tube, in which we study DNA-protein binding. Thus gene regulation studied in vitro offer only circumstantial evidence, until it is proven in vivo. To directly demonstrate binding of nuclear factors to the cis elements of genomic genes in the nucleus, in vivo DNA foot print analysis is pursued, which can be achieved by using the PCR mediated DNA foot print method.

Methods employed

Transfection assays.

To study functions of RXRβ and ICSBP we use primarily co-transfection assays. Expressible cDNAs are constructed by placing a promoter sequence in front of full length cDNA or dominant negative constructs. The promoter region of the human β-actin gene, RSV LTR or CMV enhancer (pcxn) is used to drive expression of the cloned cDNA. These expression vectors are transfected into fibroblasts, embryonal carcinoma cells, lymphocytes, and macrophages together with a test reporter gene. The reporter constructs are constructed with natural as well as artificially constructed promoters driving the fire fly luciferase gene. Various methods of transfection including the calcium phosphate precipitation method, DEAE dextran method, electroporation, lipofection are investigated for maximizing the efficiency of transfection. Following incubation for 16 to 48 hrs, cells are harvested and subjected to luciferase analysis. Transfection efficiency is normalized by cotransfected β-galactosidase activity.

In vitro transcription

In vitro transcription assays using G-free cassettes are extensively used to study how RXRβ and its heterodimer partner activate ligand dependent transcription. Thyroid hormone responsive elements such as DR-4 are connected to a 40 bp long basal promoter consisted of the TATA element and the initiation site driving a G-free cassette. Another template with a shorter G-free cassette driven by the same basal promoter without responsive elements is used as a control template. As a source of recombinant receptors, extracts prepared from recombinant RXRβ or T3R are used. The receptors and templates are mixed with nuclear extracts from B lymphocytes (containing basal transcription factors), NTPs and ligands (T3 or 9cis RA). Levels of transcription is measured by run off assays.

Genomic footprinting.

To study promoter occupancy of a genomic gene ligation mediated PCR has been extensively used in this laboratory. Cells are treated with dimethyl sulfate for 2 min and high molecular weight DNA isolated and digested with piperdine. By using an oligonucleotide primer corresponding to part of the gene the promoter sequence is extended to various positions. The extended fragments are blunt-end ligated by a common primer. This allows exponential amplification of a promoter according to methylation. The amplified fragments are resolved in a sequencing gel.

Expression

To assess a function of the regulatory genes we have isolated, it is necessary to study their expression at the level of protein. To this end monoclonal and polyclonal antibodies are raised against recombinant proteins produced in the baculovirus or in bacterial expression vectors. Mice are immunized with crude lysates containing recombinant proteins, and hybridomas producing specific antibodies are screened by positive and negative selection. Antibodies from the established hybridomas are tested for immunoreactivity with cellular proteins by using Western blot anaylysis. With these antibodies intracellular localization, tissue and developmental specificity of the expression of those immunoreactive proteins are studied. DNA binding activity of the immunoreactive materials will be tested with super mobility shift, DNA-protein blot assay and immunoprecipitation procedures.

Major findings

1. RXR in the heterodimer binds its ligand and is active in transcription. The above results are in contradiction with the report by Kurokawa et al who concluded RXR is a silent partner without transcriptional activity. These conclusions were drawn in part from the findings that RXR loses its ligand binding activity upon heterodimerization with liganded RAR. In contrast, Kersten et al () found that RXR binds its selective ligands even when heterodimerized with RAR. By differential protease sensitivity assay, we also found that each receptor in the RAR/RXR heterodimer simultaneously binds to its ligand (synthetic ligands used here were TTNPB and SR11237, respectively). Furthermore, although the RXR ligand did not stimulate ligand-dependent promoter activity alone, it dramatically synergized with RAR ligands and enhanced promoter transcription. In vivo footprinting analysis showed that only RAR ligands (but not an RXR ligand) when added alone induce footprint in the RARβ promoter. However, when an RAR ligand and RXR ligand were added together, increased stability of footprint was demonstrated. These results are consistent with the active role of RXR in the heterodimer. We

suggest that in vivo RXR/RAR heterodimers are held back fom the RARE by co-repressor(s) and additional factors, but upon ligand binding the heterodimer gains access to DNA, which triggers a cascade of factor binding to other elements and increased recruitment of basal promoters.

2. Biological effects: RAR and RXR ligands synegistically stimulate embryonic malformations, neuronal differentiation and apoptosis. Retinoids are known to cause teratogenic effects on vertebrate embryos. In collaboration with Dawid's group in NICHD, the effects of RXR and RAR selective ligands on frog (*Xenopus*) and zebrafish development were examined. In zebrafish, only the RAR ligand (but not the RXR ligand) caused embryonic malformations with the charateristic diminition of the head structure and extensive posteriorization. However, co-administration of RAR and RXR ligands significantly increased the degree of embryonic malformations both in frog and zebrafish. Interestingly, several RXR ligands added alone caused embryonic malformations similar to those seen by RAR ligands, indicating that frog embryos have a retinoid pathway distinct from otehr vertebrates.

The activities of RAR and RXR ligands were also tested on P19 cells. Retinoids induce differentiation of P19 cells into various cell types, including neuronal cells (See Horn Gudas). The RAR ligand alone (but not RXR ligand) led neuronal differentiation, as juedged by induction of neurofilament expression. However, similar to the effects seen on embryonic development, RAR and RXR ligands synergized and dramatically increased the levels of neuronal differentiation.
Although not fully documented before we found that retinodis stimulate apoptosis in a large fraction of 19 cells, seen concurrently with neuronal differentiation of otehr cells. Retinoid induced cell death was clearly due to apoptosis, as the characteristic periodic DNA ladder was formed in these cells after EA treatment. RA induced apoptosis was also confirmed with the TUNEL reaction as well. Apoptosis has been shown to occur in RA treated HL60 promeylocytic cells () and is likely to be associated with growth inhibitory activities of RA (). Apoptosis was synergistically increased when RAR and RXR ligands wer added together. These results support the active role of liganded RXR in the heterodimer formed with RAR. I would like to mention here that presumably becasue our results are at odd with the prevailing theory, we have had a difficutly in publishing some of these findings. However, our data have been internally consistent: in addition we have learned that Chambon's laboratory has observed the cooperativity by RAR and RXR and ligand depedent occupancy of the promoter (Leid, personal communication), consistent with the series of our results.

3. Construction and characterization of ICSBP-/- mice.
In collaboration with Ivan Horak in Wurzburg Germany, we have constructed ICSBP-/- mice. The standard neo-TK targeting cassette was inserted into the second exon of the ICSBP gene. Earlier analysis indicated that ICSBP-/- mice produced an aberrant ICSBP like mRNA, perhaps due to "exon skipping". We have cloned and sequenced this mRNA, and showed that it is a product of abnormal splcining and it contained multiple stop codons with no translatable sequence. Expression of abberant mRNA has been reported for IRF-1-/- mice by C. Weissmann's laboratory and STAT-1 -/- mice. The absence of the ICSBP protein in these mice

has been verified by immunoblot analysis and EMSA.

The ICSBP-/- mice exhibited a grossly abnormal phenotype in hematopoietic cells, in that the frequency of myeloid cells was dramatically increased. Spleen and lymph nodes had a large number of myeloid cells carrying the GR-1 and MacI (both markers of granulocytes). GR-1+/MacI+ cells are also markedly increased in bone marrow and peripheral blood. Microscopic observations indicated that they are typical granulocytes. In addition, GR-1-/MacI+ cells (macrophage lineage) that develop from the myeloid stem cells were moderately increased. The frequency of myeloid cells was increased even in the heterozygous ICSBP +/- mice. FACS analysis to test lymphocyte population showed that ICSBP-/- mice had T and B cells and although the frequency is reduced, the total number of T and B cells were comparable between control and ICSBP-/- mice. Some ICSBP-/- mice when they grew older (>6 months) exhibited sudden accerated meyloid cell growth, resembling a blast crisis of human myelogenous leukemia. In these mice myeloid cells invaded various nonlymphoid organs, and succumbed to earlier death. To test cytokine gene expression, qantitative PCR was performed with spleen,lymph node, bone marrow RNA. We found that expression of IL-1,IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 and IL-10, IFNα1, IFNβ, TNFα, GM-CSF, M-CSF, and G-CSF in ICSBP-/- mice is normal. However, the levels of IL-12P40 was markedly reduced in ICSBP-/- mice. Significantly, the levels of IL-12 P35, a gene encoding a smaller peptide, common in cytokines were normal. Levels of IFNγ were also somewhat lower in these animals. These results were exciting, as IL-12 produced in activated macrophages acts as a master cytokine directing T cell differentiation into TH1 cells. Because of the potent IFNγ inducing activity IL-12 is being tested for many types of infectious diseases.

We have studied infectivity of ICSBP-/- mice by various viruses. ICSBP-/- mice were capable of mounting normal resistance to VSV and mouse influenza virus, while they were much more susceptible to vaccinia virus (VV) and lymphocytic choriomeningitis virus (LCMV) than control mice and succumbed to death (control mice survived). and other studies, It has been noted that type I IFNs are required for controlling infection by VSV and influenza virus, while IFNγ and the subsequent immune responses play a major role in controlling infection by VV and LCMV. This differential requirement has been recently confirmed by analysis of knock-out mice lacking the IFNα/β receptor or IFNγ receptor gene. The differential susceptibility indicate that ICSBP-/- mice have a functional type I IFN system, but are grossly defective in mounting IFNγ dependent resistance. These results are consistent with the lack of IL-12 gene expression and the consequent deficiency in IFNγ production. Our results support the role of ICSBP in the development and function of myeloid cells.

Proposed course of work

The mechanism of TFIIB-factor interaction.

In vitro approach.

To identify molecules involved in forming a complex with TFIIB, the following in vitro assay will be deviced and the mode of interaction biochemicaly analyzed. Biotinylated oligonucleotides for the RARE or the ISRE will be immobilized to the magnetic beads, and recombinant, purified RXRβ and RARβ will be bound to the beads through the specific RARE site. Then nuclear extracts fractionated to contain different basal transcriptional activities obtained from P19 EC cells will be added to the complex in the presence or absence of TFIIB, and retinoic acid. The complex formed will be analyzed with silver staining and western blot analysis. To ensure the specificity of binding, various mutant receptors including δC2 will be used. To study the functional activity of the complex, materials will be eluted from the beads and tested in an in vitro transcription assays. As an alternative means, transcription on immobilized beads may be directly measured. Based on our previous functional data demonstrating cell type specificity, complexes formed this way are expected to contain cell type specific factors. Upon identification of specific bands we will isolate the peptides. These peptides will be further analyzed biochemically for its charge and phosphorylation property. Peptides will be microsequenced through a collaborative arrangement, and processed for cloning. Similar experiments will be performed for IRF-1 and IRF-2 as well.

In vivo approach

The most important question that can be addressed in vivo is the role of TFIID and the initiator sequence in TFIIB-factor interaction. Various mutant initiator reporters will be constructed and tested for their activity in supporting TFIIB- factor interactions in P19 and NIH 3T3 cells. We will also construct mutant TFIID (TBP) and TFIIB in expression vector and examine tehi ability to mount functional interaction. The role of USF and YY1 will be addressed as both of them are shown to be involved in initiator activity.

Analysis of ICSBP$^{-/-}$ mice:

Viral infectivity. Mice lacking the expression of ICSBP will be tested for their defense against viral infection. The following viruses will be examined: mice will be infected with murine influenza, murine herpes virus, VSV, MAIDS, and MuLV, and viral clearance, morbidity, interferon and cytokine responses as well as several parameters of immune responses will be assessed. Upon initial work with the whole animals we will infect isolated lymphocytes and macrophage populations with the respective viruses. Collaborations with several groups familiar with the above viruses are being established.

Expression of the IRF family proteins in ICSBP$^{-/-}$ mice.

Analysis of ICSBP$^{-/-}$ mice indicated that expression of IRF-2 is strongly repressed in these mice. We will study the basis of the repression by promoter analysis, western blot assays, and EMSA.

Construction of double knock-outs

We will cross IRF-2$^{-/-}$ mice with ICSBP$^{-/-}$ mice to produce double knock-outs, which will then be tested for a phenotype and viral infectivity as above.

Significance to Biomedical Research and the Program of the Institute

One of the central questions of modern biology is the mechanisms of gene regulation. This question is also the fundamental theme of developmental biology. Our program adresses the molecular basis involved in the development of the immune system. Isolation and the subsequent studies of the regulatory genes we undertook in the past year have provided not only new knowledge but also new concepts for gene regulation, which are relevant to basic biology as well as to understanding of clinical problems. Investigation of new means for dissecting the mechanisms of gene regulation pursued in this program is also consistent with overall mission of the Institute.

Bibliography

Blanco, J.C.G., Dey, A., Park, B.-Y., Minucci, S., Jurutka, P.W., Haussler, M.R., and Ozato, K. Inhibition of ligand induced promoter occupancy *in vivo* by a repressor RXRβ. Genes to Cells. 1: 209-221, 1996.

Thornton, A.M., DeVico, A.L., Buller, M.L., Wang, I-M., and Ozato, K. A dominant negative factor of the IRF family converts monocytic cells nonpermissive to HIV and vaccinia virus infection. Proc. Natl. Acad. Sci. USA., 93:383-387, 1996.

Minucci, S., Saint-Jeannet, J.-P., Toyama, R., Scita, G., Taira, M., Levin, A.A., Ozato, K., and Dawid, I.B. Retinoid receptor-selective ligands produce malformations in Xenopus embryos. Proc. Natl. Acad. Sci. USA. 93:1803-1807, 1996.

Minucci, S., Botquin, V., Sylvester, I., Dey, A., Yeom, Y.II., Zand, D.J., Ohbo, K., Ozato, K., and Schöler, H.R. Retinoic acid mediated repression of *Oct3/4* expression coincides with loss of promoter occupancy *in vivo*. EMBO.J., 15:888-899, 1996.

Nelson, N., Kanno, Y., Hong, C., Fujita, T., Fowlkes, B.J., O'Connell, E., Hu-Li, J., Paul, W.E., Jankovic, D., Sher, A.F., Coligan, J.E., Thornton, A., Appella, E., Yang, Y., and Ozato, K. Expression of Interferon Regulatory Factor (IRF) family proteins in lymphocytes. Induction of Stat 1 and ICSBP expression by T cell activation. J. Immunol., 156: 3711-3720, 1996.

Horn, V., Minucci, S., Orgryzko, V., Adamson, E.D., Howard, B.H., Levin, A.A., and Ozato, K. RAR and RXR Selective Ligands Cooperatively Induce Apoptosis and Neuronal Differentiation in P19 Embryonal Carcinoma Cells. FASEB. J. 1996. 10:1071-1077.

Yoneyama, M., Suhara, W., Fukuhara, Y., Sato, M., Ozato, K., and Fujita, T. Autocrine amplification of type I interferon gene expression mediated by interferon stimulated gene factor 3 (ISGF3). J. Biochem.120: 160-169, 1996.

Samuel, C.E., and Ozato, K. Induction of interferon and interferon-induced genes. Biotherapy. Cytokine Yearbook (Eds. Pestka, S., and H. Schellekens). Kluwer Academic Publisher (Dordrecht, the Netherlands). 8:183-187, 1996.

Hallenbeck, P.L., Minucci, S., Lippoldt, R., Phyillaier, M., Horn, V., Ozato, K., and Nikodern, V.M. Differential 9-cis retinoic acid-dependent transcriptional activation by murine retinoid X receptor α (RXRα) and RXRβ: Role of cell type and RXR domains. J. Biol. Chem. 271:10503-10507. 1996

Nunez, S.B., Medin, J.A., Givel, F., Wahli, W., Ozato, K., and Segars, J.H. The retinoid x receptor and peroxisome proliferator-activated receptor activate estrogen responsive genes independent of the estrogen receptor. J. Cell. Mol. Endocrinol (1996) In press.

Thornton, A. M., Ogryzko, V., Dent, A., Sharf, R., Levi, B., Kanno, Y., Staudt, L.M., Howard, B.H. and Ozato, K. A dominant negative mutant of an IRF family protein inhibits both Type I and Type I interferon stimulated gene expression and antiproliferative activity of IFN. J. Immunol (1996). In press.

Wang, I-M., Blanco, J., Tsai, S., Tsai, M-J., O'Malley, B. and Ozato, K. Interferon regulatory Factor-1 (IRF-1) and TFIIB cooperatively enhance interferon-responsive promoter activity in vivo and in vitro. Mol. Cell. Biol (1996). In press.

Dey, A and Ozato, K. Genomic footprinting of retinoic acid regulated promoters in embryonal carcinoma cells. Methods (Academic press). Ed: Zaret, K. In Press.

Minucci, S. and Ozato, K. Retinoid receptors in transcription. Curr. Op. Gen. Dev. 1996, In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 08719-16 LMGR

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development and Uses of Eukaryotic Vectors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. :	Bruce H. Howard	Chief	LMGR, NICHD
Other:	E. Brinkmann	Techinician	LMGR, NICHD
	E. Englander	Visiting Associate	LMGR, NICHD
	K. Engleka	Visiting Fellow	LMGR, NICHD
	G. Humphrey	Visiting Associate	LMGR, NICHD
	V. Ogryzko	Visiting Fellow	LMGR, NICHD
	V. Russanova	Visiting Fellow	LMGR, NICHD

COOPERATING UNITS *(if any)*

NCHGR, NIH (Eric Green)
NICHD, NIH (Yoshihiro Nakatani)

LAB/BRANCH

Laboratory of Molecular Growth Regulation

SECTION

Section on Human Cell Genetics

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-2753

TOTAL STAFF YEARS: 7	PROFESSIONAL: 6	OTHER: 1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cell cycle regulation in normal, non-immortalized cells, e.g., human or mouse fibroblasts, remains poorly investigated. Recent evidence suggests that the progression of such cells into a post-mitotic state ("senescence") or to the alternative fate, immortalization, may reflect a drift in differentiation state which is responsive to gene products that act on higher order chromatin structure. To investigate this possibility, two histone deacetylase genes (one of which was isolated by this group) have been introduced into human fibroblasts using a newly developed retroviral vector system. In related work, genes encoding two histone acetyltransferases, hGCN5 and hP/CAF, were observed to inhibit G1 to S phase transit in HeLa cells. These histone acetyltransferases are likewise being introduced by retrovirus-mediated transduction into human fibroblasts. To examine whether alterations in higher order chromatin structure accompany cell senescence or immortalization, a new method was developed to map euchromatin vs. heterochromatin domains. Application of this technique in the context of "genomic differential display" revealed for the first time loci which switch chromatin state in association with human fibroblast senescence.

PHS 6040 (Rev. 5/92)

The **Human Genetics Section**, under the direction of Bruce Howard, investigates mechanisms underlying cellular immortalization and its converse, cellular senescence. Cellular senescence in human diploid fibroblasts (HDF) and other human cell types has been widely studied, yet in many aspects remains poorly understood. One area which in particular merits further investigation is the apparent relatedness of cellular senescence and terminal differentiation. Although the two processes are not necessarily synonymous, in many respects senescence resembles an aberrant drift in differentiation state, with cells acquiring a phenotype that is suboptimal for tissue function and maintenance. HDF proliferative potential is primarily dependent on the number of rounds of DNA synthesis completed rather than the cumulative time in culture; thus, to the extent that senescence is considered as akin to terminal differentiation, it most closely resembles model systems (e.g., murine erythroleukemia or promyelocytic leukemia cells) where a requirement for passage through the cell cycle has been demonstrated.

Histone acetylases and deacetylases. In previous work, Howard and his coworkers demonstrated that either of two histone deacetylase inhibitors, sodium butyrate or trichostatin A, could dramatically reduce HDF proliferative lifespan. Intriguingly, reduced proliferative potential was observed to be dependent on one or more cell doublings in the presence of these agents. Cells arrested and subsequently released from histone deacetylase inhibitors displayed markers of senescence and exhibit a persistent G1 block, but remained competent to initiate a round of DNA synthesis in response to SV40 T antigen. Average telomere length in prematurely arrested cells was greater than in senescent cells, reflecting a lower number of population doublings completed by the former. Taken together, these results support the view that one component of HDF senescence mimics a cell cycle-dependent drift in differentiation state, and that propagation of HDF in histone deacetylase inhibitors accentuates this component.

Several lines of evidence point to histone deacetylases as specific targets of trichostatin A. Nevertheless, to pursue these findings further, the next step for Howard and his group was to confirm that genetically altering the balance of histone acetyltransferases and deacetylases could perturb fibroblast proliferative potential in a manner consistent with the results obtained with trichostatin treatment. Based on expressed sequence tags (ESTs) in the Human Genome Database, a second human histone deacetylase encoding gene, hHD2, was isolated and characterized by DNA sequence analysis. Over a 300 amino acid N-terminal conserved region, hHD2 shares 66% identity at the amino acid level with hHD1, the first human histone deacetylase identified. Interestingly, the level of conservation is similar when hHd2 is compared with the Saccharomyces cerevisiae histone deacetylase gene, yRPD3. In the latter case, there is 65% identity at the amino acid level between the two genes. For each of these histone deacetylases, the C-terminal region is divergent. What effect(s) the divergent C-terminal domains have on function remains to be determined.

In concurrent collaborative work, the Howard group investigated growth regulatory activities of two histone acetyltransferases, hGCN5 and hP/CAF. The cloning and characterization protein-protein interactions in which these histone acetyltransferases participate is described in greater detail in an accompanying section by Nakatani and his coworkers. hP/CAF was introduced into HeLa cells by DNA-mediated transfection, and its effect on cell cycle distribution assessed by affinity sorting of tranfected cells in conjunction with dual parameter flowcytometry. P/CAF caused a reproducible redistribution of cells from S phase to G1 phase in this assay. In parallel experiments to confirm the validity of this experimental protocol, plasmids encoding E2F-1, simian virus 40 small-t antigen, cyclin A or clyclin E increased the accumulation of cells in S phase, whereas plasmids encoding the cyclin-dependent kinase inhibitor p21 or p27 reduced the number of S-phase cells. hGCN5 was likewise evaluated by the HeLa transfection assay. Although at least ten-fold higher expression levels were achieved than with hP/CAF, only a modest hGCN5-mediated redistribution of cells from S phase to G1 phase was observed.

Based on evidence that the adenovirus-encoded oncogenic protein E1A and hP/CAF compete for binding sites on p300, it was considered that cotransfection of hP/CAF with E1A would oppose the mitogenic activity of the E1A. E1A alone had mitogenic activity in HeLa cells, whereas an E1A mutant lacking a p300-binding domain exhibited very weak activity. When hP/CAF was cotransfected with E1A, the mitogenic activity of E1A was significantly counteracted by P/CAF. These results support the view that P/CAF and E1A mediate antagonistic effects on cell cycle progression.

Retrovirus-mediated transduction. The preceding experiments were performed using an immortalized tumor cell line. Surprisingly little information exists concerning growth regulation in non-immortalized cells, since gene transfer into representative populations of such cells has proven to be difficult. To rectify this situation, Howard and his coworkers developed a retrovirus-based system that incorporates the Tac antigen (IL-2 alpha receptor subunit) affinity cell sorting tag in a bicistronic configuration. The system is so designed that gene coding regions of interest can be inserted upstream under control of the Moloney leukemia virus promoter, thereby achieving maximum expression levels. Expression of the surface tag is tightly linked, since translation occurs via an internal ribosome entry signal (IRES) on the same mRNA that encodes the upstream gene. In experiments using optimized viral infection protocols, newborn mouse skin fibroblasts were stably transduced with an efficiency of 25%, while human skin fibroblasts were transduced at a level of 15%. Of note, viral supernatants in such experiments were generated by transient transfection of amphotropic BING packaging cells. Affinity cell sorting experiments revealed that stably transduced populations could be enriched from about 15% to greater than 90% in a single step without cell toxicity. With these techniques in place, facile genetic manipulation of growth, cell cycle, and gene regulation in non-immortalized cells should be possible.

Higher order chromatin structure. Histone deacetylase inhibitors can either induce or block induction of gene expression, depending on the target gene studied. In cases where transcription is activated, the mechanism is thought to involve hyperacetylation of histones H3 and H4, likely facilitating enhancer function. Importantly, histone hyperacetylation and transcriptional effects are reversible after these inhibitors are removed. The simplest way to reconcile the reversibility of histone deacetylase inhibitors (i.e., on acetylation and local transcription) with the apparently irreversible cell cycle exit that these agents can induce is to postulate that cell doubling in the presence of these agents alters epigenetic silencing mechanisms within the cell. This predicts that changes in higher order chromatin structure will be observable in association with progression of non-immortalized cells towards the post-mitotic state termed senescence. The proposal that alterations in epigenetic silencing may be linked to senescence is not without precedent. Correlative evidence consistent with selective heterochromatin instability as a contributory factor in senescence derives from reports of age-dependent recession of a subset of heterochromatin domains both in vivo and in vitro. Results from a studies on aging in *Saccharomyces cerevisiae* have also led to the suggestion that the stability of repressive chromatin domains may be a strong determinant of proliferative potential.

To map higher order chromatin structure, Howard and his collaborators developed and applied a method in which chromatin fragments are immunofractionated with affinity purified anti-acetylated histone H4 antibody. In this assay, euchromatin, in which histone H4 is moderately or highly acetylated, distributes preferentially into the antibody-bound fraction, whereas heterochromatin, in which histone H4 is underacetylated, distributes preferentially into the unbound fraction. The chromatin state at single copy loci can be assessed by quantitative PCR using appropriate sets of primers. For euchromatic loci, PCR products are detected primarily in the bound fraction, while the converse is observed for heterochromatic loci. In complementary work, replication timing was measured at specific loci. In this second assay, euchromatic regions typically replicate early in S phase, while heterochromatic regions replicate late in S phase.

Chromatin in the chromosome 7q subtelomeric region. The human genomic region chosen initially for analysis higher order chromatin structure corresponded to a 1.7 megabase yeast artificial chromosome (YAC) contig spanning the most centromere-distal subtelomeric segment in chromosome 7q. PCR primers which had been designed to define sequence tagged sites (STSs) in the Human Genome Project were utilized to sample chromatin structure at loci arrayed across this region. The terminal 100 kilobases adjoining the telomere were examined at STS loci spaced about 20 kilobases apart. These experiments revealed the presence of multiple interspersed euchromatic and heterochromatic domains at the chromosome 7q terminus. While the most distal 300 kilobases appeared to be predominantly heterochromatic in nature, euchromatic domains were detected at 200 kilobases from the telomere (corresponding to a CpG island) and at about 25 kilobases from the telomere. No comparable information on higher order chromatin structure near a human chromosome terminus has been reported previously.

Epigenetic drift detected by genomic differential display. In order to obtain an unbiased view of higher order chromatin structure in normal human fibroblasts, Howard and his coworkers went on to establish a novel genomic differential display method comparing anti-acetylated histone H4 bound and unbound chromatin fractions. Using this method, thousands of loci can be rapidly screened to identify those with strongly euchromatic or heterochromatic characteristics, or more interestingly, a predisposition to shift from one chromatin state to the other. Of the first approximately one thousand loci examined, three were found to shift from representation predominantly in the heterochromatin (unbound) to the euchromatin (bound) fraction. These loci appear to represent regions of instability in epigenetic silencing.

Conversely, four were observed to shift from the euchromatin to the heterochromatin fraction. To the extent that this latter result reflects de novo silencing events, it has intriguing implications with respect inactivation of tumor suppressor genes and thus the drift of non-immortalized cells in culture towards a decision point between senescence or immortalization.

Further studies on loci manifesting epigenetic instability will focus on sequence analysis, cloning and determination of chromatin structure over larger contiguous genomic regions, mapping with respect to chromosome localization, and searches for nearby genes whose expression show changes consistent with chromatin structure alterations. Ultimately, such loci, when monitored in conjunction with genetic manipulations as described above, should provide the means to investigate controls on epigenetic stability in mammmalian cells.

OSD

NICHD Annual Report
October 1, 1995 to September 30, 1996

Laboratories of the Scientific Director

DNA REPLICATION, REPAIR, AND MUTAGENESIS

This Section, directed by ***Arthur Levine***, is focused on the molecular mechanisms of DNA replication, repair, and mutagenesis, with work in both eukaryotic and prokaryotic models. It is evident that genetic disorders, birth defects, and cancer are all consequent to aberrant DNA replication, mutations, or other lesions that compromise the molecular anatomy of the genome. However, genomic fidelity also has implications for evolution: No mutations mean no evolution, but too rapid a mutation rate means that a species will quickly cease to exist. Normally, DNA replication is a highly accurate process: In *Escherichia coli,* only one error occurs in every 10^{10} bases replicated. Much remains to be learned about the mechanisms that maintain this remarkable degree of fidelity.

Mutagenesis in Bacteria: the *umuDC* Operon. During the past year, ***Roger Woodgate*** has continued to work on a prokaryotic mutagenesis system, of particular interest since the genes that promote mutagenesis in bacteria have been much more clearly defined than those in eukaryotes. When *E. coli* is treated with DNA damaging agents, e.g., ultraviolet radiation (UV), the RecA "super protein" is activated; activated RecA (RecA*) then mediates the proteolytic cleavage of LexA protein, the repressor of the multi-gene "SOS" pathway. This results in the induction of a number of genes whose products are directly or indirectly involved in DNA repair and mutagenesis. Generally, the organism first attempts to repair DNA lesions in an error-free manner. However, if this pathway fails, a second component of the SOS system, the tightly repressed *umuDC* operon, is induced, and the products of the *umuDC* genes allow the DNA replication machinery to bypass unrepaired sites of damage. This "trans-lesion" synthesis is, however, error-prone: Mutagenesis may be the cost of continued survival (of course, some mutations could promote survival). DNA polymerase III holoenzyme, RecA*, and the UmuDC proteins are all directly required for mutagenic trans-lesion synthesis, but the actual mechanism by which the UmuDC and RecA* proteins promote mutagenesis is unknown. In previous work, Woodgate et al. found that the *E. coli* UmuD protein must be proteolytically processed to its active, truncated form, UmuD′, and this cleavage is mediated by RecA* (which wraps its filament around damaged DNA). Genetic experiments had also suggested that RecA* physically interacts with the Umu proteins. Recently, this group found direct proof of a physical association between RecA* and UmuD′, suggesting a mechanism by which the Umu proteins could be specifically targeted by RecA* to lesions in DNA. Thus, this protein-protein-DNA targeting interaction is a third role of RecA in mutagenesis (cleavage of the LexA repressor of *umuDC* and UmuD cleavage being the other two roles).

To increase our understanding of the mutagenic process, this group has sought to determine the molecular structure of the mutagenically active *E. coli* UmuD′ protein. During the past year, this protein was overproduced, purified, and crystallized by the hanging drop method. The structure was refined to 2.5 Å and analyses revealed that the protein adopts an apparently novel fold, in that it is a tight beta structure with an extended amino terminus and a single alpha helix encompassing residues 39-45. The seven beta strands are connected by short loops, and are oriented to form three beta sheets, which form the main body of the protein. The crystal is formed by two different kinds of protein interactions. In addition to UmuD′ forming a molecular dimer with itself, the amino terminal of UmuD′ can interact with the amino terminal of another "molecular dimer" to form an extended polymer ("filament") structure. While deletion of the N-terminal of UmuD′ still allows the protein to form the "molecular dimer", it precludes the formation of the polymer and greatly reduces the ability of UmuD′ to bind to the RecA nuclear protein filament, explaining why cells expressing this mutant are nonmutable. During the past year, this group has also sought to overproduce and purify UmuC as well as the UmuD′C complex, so that the complete structure of the "mutasome" can be elucidated. Analysis of the DNA binding properties of the purified UmuD′C complex revealed that the complex does not bind to ds DNA, but does so cooperatively to ss DNA. Moreover, these results suggest that prior binding of UmuD′C to DNA may inhibit the extent to which RecA can form a nucleoprotein filament. Further experiments were also carried out this year on UmuC: Unlike *E. coli*, the closely related bacterium *S. typhimurium* is poorly responsive to the mutagenic effects of DNA damaging agents. In an attempt to explain this difference, chimeric UmuC proteins were constructed in which various domains encoding the UmuC protein were exchanged between *E. coli umuC* and *S. typhimurium umuC*. These constructs revealed that alterations in the region located between *S. typhimurium* UmuC residues 26-59 are the most likely cause for the non-mutable phenotype.

Woodgate and his coworkers have also investigated the *in vivo* stability of the Umu proteins in *E. coli*, with the finding that UmuD and UmuC proteins are inherently unstable in this organism and are degraded by the *Lon* protease. However, UmuC is protected when coexpressed with UmuD′. Interestingly, while the homodimeric UmuD′ protein is quite stable, it is rapidly degraded by the *ClpXP* protease when in a heterodimeric complex with UmuD. Given that UmuD normally exists in a much higher concentration than UmuD′, and that an equilibrium is maintained between the heterodimer and the homodimer, it stands to reason that the homodimer exists only under conditions of severe cellular stress and that otherwise, formation of the heterodimer (which is exquisitely sensitive to proteolytic degradation) is favored. Thus, the induction of the $UmuD'_2C$ complex is under very tight control and once produced, the complex only exists long enough to carry out "last resort" translesion DNA synthesis – but no longer – avoiding gratuitous mutagenesis.

Eukaryotic Mutagenesis Pathways. During the past year also, studies were undertaken with *Xenopus laevis* cells in an attempt to learn whether a mutagenesis pathway, similar to the Umu pathway, exists in eukaryotes. Closed circular ss DNA is efficiently replicated to ds DNA in *Xenopus* oocytes, eggs, and nuclear extracts, but Woodgate and his collaborators found virtually no DNA synthesis when UV-irradiated circular ss DNA, containing as few as 2-3 cyclobutane dimers, was injected in oocytes. The replication arrest operates only in *cis*, i.e., cellular DNA synthesis is not arrested when damaged template molecules are injected. Interestingly, oocytes that had been unable to replicate irradiated ss DNA were able to replicate this substrate when it was injected after the oocytes had been matured with progesterone. Thus, the replication arrest is abrogated during meiosis, suggesting the emergence in matured oocytes of a replicative bypass pathway. Since the Umu proteins are thought to act by facilitating translesion DNA synthesis in prokaryotic cells, these workers sought to determine if they would perform similarly in immature frog oocytes. Indeed, micro-injected mRNAs encoding *E. coli* UmuD′ and UmuC did promote significant replication of irradiated ss DNA, reflecting a remarkable conservation of prokaryotic to vertebrate replication machinery.

True *umu*-like genes have been found in a variety of gram-negative enteric bacteria, and to date, this lab and others have identified eight closely related homologs of the *E. coli* UmuC protein, with all appearing to play critical roles in damage-inducible mutagenesis in *enterobacteriaceae*. During the past year, this lab also identified a distantly related UmuC homolog, *DinB*, in *E. coli*, as well as a new member of the UmuC-superfamily in the archeon, *Sulfolobus solfataricus* [*dbh* (*dinB* homolog)]. Other newly identified homologs include a *C. elegans* protein (most closely related to yeast REV1), as well as two human ESTs putatively related to the UmuC-like proteins. Thus, the evidence is strong that a Umu-like pathway has been highly conserved structurally and/or functionally – and may even be present in human cells.

Spectra of Mutations Generated by the Umu System and its Homologs. The eight *umu*-like operons identified to date in *enterobacteriaceae* are structurally similar, but they differ in their ability to enhance cellular and bacteriophage mutagenesis. The specificity of the mutator phenotype of a *recA730 lexA51*(Def) strain was analyzed by employing F′-plasmids carrying a set of mutant *lacZ* genes that can individually detect specific transitions, transversions, and frameshift events. Most of the spontaneous mutagenesis could be attributed to a specific increase in A:T→T:A, A:T→C:G, and G:C→T:A transversions. These events were completely abolished in a Δ*umuDC* strain, indicating that functionally active UmuD′C proteins are normally required for their generation. The spectrum obtained was similar to that of strains with a defect in the ϵ (3′-5′ proofreading) subunit of DNA polymerase III, raising the possibility that the wild-type ϵ protein is inactivated in strains expressing the RecA730 and UmuD′C proteins. An ss DNA vector carrying a site-specific T-T cyclobutane dimer was also used to analyze the mutagenic specificity of a set of isogenic *E. coli* Δ*umuDC* strains harboring low-copy-number plasmids expressing UmuD′C or its plasmid homologs (*mucA′B, rumA′B*). In *umuD′C* strains, 3′ T → A mutations outnumbered 3′ T → C, but the reverse was true for the homologs, suggesting that UmuD′C and its homologs may differ in their relative abilities to promote elongation from mismatched termini. (The enhanced frequencies of UV-induced mutations observed in strains expressing the *mucAB* or *rumAB* genes do not appear to result from any general increase in the error-rate of translesion synthesis, but are more likely explained by their superior abilities to promote translesion synthesis.)

In the coming year, this group will seek to refine its structural model of the "mutasome" (i.e., the complex that the Umu proteins make not only with each other and with RecA*, but with the subunits of DNA polymerase). A determination of the structure of the mutasome in its entirety will now be feasible, given the success of this group in purifying a soluble UmuD′C complex as well as the individual proteins. Work will continue on a eukaryotic homology search, both structural and functional, and studies will also continue on the factors that control the stability of the "mutasome". Clearly, both the initiation and duration of mutagenesis are under very tight control, with multiple levels of regulation, as befits a pathway with such critical consequences for survival and genome integrity. During the next year also, further studies in the *Xenopus* oocyte system will be undertaken to determine if the bypass synthesis seen in this system is in fact mutagenic. If so, additional proof that the molecular mechanisms of mutagenesis are remarkably conserved will be at hand. Beyond the studies in the *Xenopus* system, the focus will be on the putative UmuC-like homolog recently identified in yeast, given that DNA repair mechanisms in general appear to have been highly conserved between yeast and humans. Finally, with soluble UmuD′C in hand, this group will now focus on the interactions of the Umu proteins with various subunits of DNA polymerase III in order to determine the precise molecular mechanisms that facilitate translesion DNA synthesis.

Mammalian DNA Repair: A DNA Damage-Specific, DNA Binding (DDB) Protein. DNA repair is a multi-step process which removes structural lesions from genomic DNA. If not repaired, DNA lesions can lead to mutations and/or alteration of gene expression, and ultimately, to cell transformation or death. The significance of the DNA repair process for human health is well reflected in the rapidly increasing number of human visceral cancers now known to arise as a consequence of mutations in repair genes (or genes that normally prevent mutagenesis), as well as the heritable human skin disease, xeroderma pigmentosum (XP). The high susceptibility of XP patients to sun-induced skin cancer is strongly correlated with the hypersensitivity of their cells to UV light, and the decreased capacity of these cells to repair UV-induced photoproducts in DNA. Efforts to clone the genes that are defective in the various XP complementation groups and to identify their products, which might provide the key to a fuller understanding of normal repair processes in human cells, are only now meeting with success.

A group led by ***Arthur Levine*** has used model DNAs containing UV-induced lesions to address the molecular mechanism of lesion recognition. When mammalian cells are pre-treated with low-dose UV, the repair of transfected DNA, damaged *in vitro* by UV, is enhanced. This group previously identified a constitutive DNA damage-specific, DNA binding (DDB) protein complex in primate cells which is induced to high levels of activity by this pre-treatment. The DDB complex appears to recognize a specific UV-photoproduct (6-4 pyrimidine dimers); it is the first such damage-specific, damage-inducible DNA binding complex to be identified in mammalian cells. The M_r of the largest DDB subunit is ~127 kDa; a 48 kDa protein is present as well. The function of the DDB protein complex *in vivo* is still uncertain. However, it is likely that the complex is utilized in some aspect of processing of UV-damaged DNA, probably the initial recognition of damage, since its activity is regulated by UV light, it has no catalytic activity, and it is absent in the cells of some XP (Group E) patients. Moreover, DDB activity is highly conserved among vertebrates (e.g., it is found in chickens, marsupials, rodents, and primates), suggesting an important function in higher eukaryotes.

Previously, by exploiting DDB's high affinity for UV-irradiated DNA, these workers used DNA affinity chromatography to obtain highly purified primate p127 protein. The protein was microsequenced, the p127 cDNA was cloned, and the human p127 gene was assigned to chromosome 11. A p127 homolog was also found in *Drosophila*, which should allow this group to exploit the power of fly genetics to elucidate the function of p127. Other proteins that may interact with p127 to form the DNA lesion-recognition complex, especially the p48 protein, have now been identified and sequenced. During the past year, this group found that DDB activity (i.e., DNA binding) is detectable in both the nucleoplasm and nuclear pellet of primate cells, but not the cytoplasm. Immediately after exposure to UV light, DDB activity can be detected after high salt extraction in the nuclear pellet but not in the nucleoplasm. These results suggest that the DDB protein complex is translocated from a loose association within the nucleus to a tight association, presumably with UV-damaged DNA. In the past year also, using cell fractionation and specific antibodies, these workers examined the nuclear distribution of p127 and several other nucleotide excision repair (NER) and DNA replication proteins before and after UV treatment of human cells. Following irradiation, the p127 component of DDB, as well as XPA, RP-A, and PCNA, are translocated from low to high salt chromatin, suggesting again that these proteins move to a tight association with damaged DNA. However, unlike XPA (which functions in the early recognition of various DNA lesions), only a small fraction of p127 protein is translocated to the high-salt chromatin. This result suggests that DDB may have another cellular function(s) in addition to its role in NER. Moreover, there appears to be a specific interaction between p127 and RP-A, both *in vitro* and *in vivo*. The DDB/RP-A interaction results in enhanced binding of both proteins to DNA, and the similarity of DDB/RP-A to XPA/RP-A interactions additionally supports the notion that DDB functions in the damage-recognition step of NER.

In other recent studies, the purified primate DDB complex was added to an *in vitro* reconstituted NER system, with the finding that the purified complex does stimulate NER modestly, although it does not appear to be an essential component of that system; rather the DDB complex may play an auxiliary role in recognition, i.e., this complex may specifically recognize and bind to 6-4 photoproducts with higher affinity than the XPA-mediated recognition of diverse DNA distortions. It is also possible that DDB participates in the catalytic turnover of the incision/excision NER complex during repair of UV-irradiated DNA. Studies in the coming year will focus on the two issues described above: A possible role for the DDB complex in addition to damage-recognition, e.g., transcription coupling, and the specific molecular function of this complex in NER *per se*.

SECTION ON DNA REPLICATION, REPAIR, AND MUTAGENESIS

Chief
Arthur S. Levine, M.D.

Principal Investigator
Roger Woodgate, Ph.D.

Post-Doctoral Fellows
Ekaterina Chumakov, Ph.D.
Martin Gonzalez, Ph.D.
John McDonald, Ph.D.
Vesna Rapić Otrin, Ph.D.
Olga Studitskaia, Ph.D.
Dominique Vandewiele, Ph.D.

Pre-Doctoral Fellows
Erinn Maury
Kevin Winterling

Guest Researcher
George Kopsidas, Ph.D.

Senior Technical Specialist
Janet Hauser

For Branch Chief's Summary: NERVE GROWTH FACTOR

The Section on Growth Factors, under the direction of Gordon Guroff, has as its prime objective the study of nerve growth factor (NGF) at the biochemical, molecular, and cellular level and the mechanism(s) by which it and the other neurotrophins control the survival, development, and death of key neuronal populations. Nerve growth factor was the first recognized and is now the best characterized member of the neurotrophin family of peptide growth factors. The neurotrophin family, now five in number, supports a wide variety of neural cells. Nerve growth factor is required for the survival and development of sympathetic and sensory neurons. It also is involved in the support of several other cell types, including specific populations of neurons in the central nervous system, the cells of the adrenal medulla, and a number of tumors as well. The action of nerve growth factor on these different cells is initiated by its binding to specific receptors. There are two separate nerve growth factor receptors, one the product of the trk protooncogene and a second, lower-affinity site known as p75. The binding of nerve growth factor to these receptors activates one or more signal transduction pathways that lead to alterations in the phosphorylation and, consequently, the function of key proteins in the cell and to changes in the expression of specific genes. These changes in protein function and in gene expression, caused by the changes in phosphorylation, are the mechanism by which nerve growth factor exerts its effects on its target cells. Much of the work leading to this concept has been done with the PC12 pheochromocytoma, a cell line derived from a tumor of the rat adrenal medulla. This clonal line continues to be one of the most informative tools available for the study of nerve growth factor and a key model for neuronal differentiation in general. In the presence of nerve growth factor, PC12 cells stop dividing, elaborate neurites, become excitable, and will synapse with appropriate muscle cells in culture. Indeed, they change from a rapidly-dividing chromaffin cell to a terminally-differentiated sympathetic neuron within a few days. The changes in phosphorylation that underlie these striking alterations in phenotype occur in virtually every compartment in the cell. Phosphorylation of nerve growth factor-stimulated calcium channels appears to regulate the calcium flux across the membrane and, in turn, the intracellular calcium levels. These levels are involved in neuronal survival, neuronal protection, and synaptic plasticity. Nerve growth factor-induced phosphorylation of the elements that control protein synthesis, such as eIF-4E, eEF-2, and S6, almost certainly alters the rate and the specificity of transcription of specific genes and the translation of the mRNAs that are produced from them. The nerve growth factor-induced phosphorylation of specific transcription factors determines which genes are expressed. An understanding of the action of nerve growth factor will surely illuminate the control of neuronal differentiation and survival. Clinical interest in these peptide factors is intense because of the role they might play in neurodegenerative diseases.

Nerve Growth Factor and Calcium Uptake. Calcium uptake into PC12 cells is increased by treatment of the cells with nerve growth factor. Furthermore, this action of nerve growth factor depends upon a unique calcium channel and the stimulating action of nerve growth factor on this channel is probably due to a phosphorylation of one or more of the channel subunits. It has been suggested that, since the survival of neurons requires adequate intracellular calcium levels, this action may be one of the mechanisms by which nerve growth factor keeps neurons alive. Present work focusses on the pathway by which this channel is activated and recent data implicate protein kinase C. Long-term down-regulation of kinase C with the tumor promoter PMA prevents the action of nerve growth factor on calcium uptake, and the use of isoform-specific effectors has identified kinase Cα as the kinase isoform involved in nerve growth factor action. The action of the kinase inhibitor, K-252a, previously shown also to simulate calcium uptake into PC12 cells, is also prevented by down-regulation of kinase C and the same isoform-specific effectors that influence nerve growth factor action also influence the action of K-252a, suggesting that these agents act through one or more common cellular elements, perhaps the nerve growth factor receptor itself. Related

studies with 3T3 cells transfected with nerve growth factor receptors show that both the high-affinity nerve growth factor receptor, p140^{trk}, and the low-affinity nerve growth factor receptor, p75^{NGFR}, will mediate nerve growth factor-stimulated calcium uptake into PC12 cells, but perhaps by different mechanisms. This work highlights the role of calcium in the action of nerve growth factor and, in turn, the role of nerve growth factor in neuronal survival, neuronal protection, and neuronal plasticity.

Nerve Growth Factor and S6 Phosphorylation. A continuing interest in the nerve growth factor-stimulated phosphorylation of the ribosomal protein S6 has led, in previous work, to the identification of a unique 47 kDa nerve growth factor-stimulated S6 kinase in the cytoplasm of PC12 cells. It now appears that this new kinase is related immunologically to the p70^{S6K} present in many cells. Not only do antibodies directed against p70^{S6k} recognize the 47 kDa enzyme, but rapamycin, an inhibitor shown to inhibit the growth factor-induced activation of p70^{S6k} in whole cells, inhibits the nerve growth factor-induced activation of the 47 kDa enzyme in PC12. Another interesting development has been the finding that the 47 kDa S6 kinase is rather specifically bound to ribosomes. Indeed, when compared to the other enzymes known to phosphorylate S6 in the cells, the 47 kDa kinase is bound in much greater proportion than the others. Although it is clear that in order to phosphorylate a ribosomal protein, these kinases must bind to the ribosomes at least transiently, the observation of the comparatively tighter binding of the 47 kDa kinase opens the door to studies on the specific binding sites on both kinase and ribosome. The study of the S6 kinases in PC12 cells has important implications for the action of nerve growth factor on protein synthesis and, in turn, on the phenotypic changes nerve growth factor induces in its target cells.

Nerve Growth Factor and the Transcription Factor NGFI-B. The transcription factor NGFI-B is induced by a number of different effectors in PC12 cells. Nerve growth factor is among these, but nerve growth factor has the added effect of inducing a robust phosphorylation of the molecule. Previous work from this laboratory has shown that one specific nerve growth factor-induced phosphorylation has the effect of inhibiting the binding of NGFI-B to its target sequence on DNA and, presumably, limiting its transcriptional activity. A kinase, termed NGFI-B kinase I, has been purified and characterized and it has been shown to phosphorylate a specific serine in the DNA-binding domain of NGFI-B. The identity of this kinase is not known for sure, but it is very similar or even identical to CREB kinase, the enzyme phosphorylating the protein that binds to the cAMP response element. In order to understand the biology of this apparently anomalous action of NGFI-B kinase, e.g., an enzyme induced by nerve growth factor that blunts the action of a transcription factor also induced by nerve growth factor, experiments have been done with other effectors that induce NGFI-B, but do not induce NGFI-B phosphorylation. It has now been shown that the induction of NGFI-B by depolarizing concentrations of K^+ causes increased transcription of a reporter gene linked to the NGFI-B response element. However, the addition of nerve growth factor as well inhibits this action of K^+. These data confirm the in vivo role of NGFI-B kinase, a role hitherto shown only in a cell-free system. They also highlight the importance of the nerve growth factor-dependent induction of NGFI-B kinase rather than of NGFI-B itself. Perhaps most important, they provide an example of the interaction of nerve growth factor with neuronal activity in the expression of specific genes. One interpretation of the data could be that at a certain developmental stage, perhaps upon synapse formation, the role of neuronal activity in gene expression is altered by the availability of agents such as nerve growth factor.

Nerve Growth Factor and the Epidermal Growth Factor Receptor. The finding, some years ago, that PC12 cells respond both to the differentiating agent nerve growth factor and the mitogen epidermal growth factor, has led to important studies on comparative signal transduction and to equally significant experiments on receptor interplay. The latter are based on the finding that differentiation with nerve growth factor causes the down-regulation of the epidermal growth factor receptor. In an effort to understand the molecular basis of this down-regulation, work in the Section has focussed on a possible nerve growth factor-induced decrease in the translation of the epidermal growth factor receptor mRNA. Two different translation systems have been used, one a fortified rabbit reticulocyte lysate and the other an S10 system from the PC12 cells themselves. Using these systems it has been possible to translate epidermal growth factor receptor mRNA. Over the past year, constructs have been made that contain several hundred bases of the sequences flanking the epidermal growth factor receptor message linked to the reporter gene luciferase. These constructs are now being transcribed and translated in cell-free systems from naive and nerve growth factor-treated PC12 cells in order to determine, first, the influences that regulate epidermal growth factor receptor translation and, second, the involvement of nerve growth factor in that regulation. Also, the levels of epidermal growth factor receptor mRNA have been measured by competitive RT-PCR, and nerve growth factor-induced decreases in mRNA level have been seen for the first time. Efforts are underway to measure epidermal growth factor receptor mRNA stability as well as to elucidate the elements involved in its formation. In a related line of research, the signal transduction pathway controlling this nerve growth factor-induced down-regulation has been sought. Using PC12 cells transfected with dominant- negative signaling elements, it has been shown that the ras-erk pathway is responsible for this down-regulation as it is for neurite outgrowth itself. However, it is clear that epidermal growth factor receptor regulation is not linked to neurite outgrowth, as it occurs in nerve growth factor-treated cells that are prevented from attaching to a matrix and it does not occur in cells treated, for example, with staurosporine, which make neurites but, as shown by recent data from this laboratory, by a different signal-transduction pathway than that serving nerve growth factor-induced neurite formation. These studies give promise of elucidating the molecular mechanism by which the levels of epidermal growth factor receptor are controlled during nerve growth factor-induced neurite formation. Parallel studies suggest that both erbB2 and erbB3, receptors, that are homologous with the epidermal growth factor receptor and are of significant clinical interest because of their possible role in the initiation or promotion of tumor growth, undergo similar nerve growth factor-induced down-regulation and that their levels may be controlled by mechanisms similar to those regulating the levels of the epidermal growth factor receptor.

SECTION ON GROWTH FACTORS-FY96

Chief, Gordon Guroff, Ph.D.

Post-Doctoral Fellows
Hao Jiang, Ph.D.
Yasuhiro Katagiri, Ph.D.
Mariam Monshipouri, Ph.D.
Makoto Shibutani, Ph.D.
Michael Whalin, Ph.D.
Elena Kazanova, Ph.D.
Michael Czar, Ph.D.

Guest Researchers
Philip Lazarovici, Ph.D.
Vilen Movsesyan, Ph.D.

Senior Technical Specialist
Geneva Dickens

Project Numbers - Annual Report 1996

OFFICE OF THE SCIENTIFIC DIRECTOR (OSD)

ZO1 HD 00093-22	Mechanism of Action of Nerve Growth Factor Gordon Guroff, Ph.D.
ZO1 HD 01500-14	Studies on DNA Replication, Repair, and Mutagenesis in Eukaryotic and Prokaryotic Cells Arthur S. Levine, M.D.

ZO1 HD 01502-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00093-22 OSD

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of Action of Nerve Growth Factor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Guroff, Ph.D.	Head, SGF	OSD, NICHD

Others: (See Attached)

COOPERATING UNITS *(if any)*

(See Attached)

LAB/BRANCH

Office of the Scientific Director

SECTION

Section on Growth Factors

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
11.75	9.25	2.50

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Nerve growth factor (NGF) was the first recognized and is now one of the best characterized of the peptide growth factors. It is a member of a family of homologous growth factors, called the neurotrophins, now five in number, that supports a wide variety of neural cells. NGF is required for the survival and development of sympathetic and sensory neurons. It also is involved in the support of several other cell types, including specific populations of neurons in the central nervous system, the cells of the adrenal medulla, and a number of tumors as well. The action of NGF on these different cells is initiated by its binding to specific receptors. These are made up of two separate proteins, the product of the trk protooncogene and a lower-affinity site known as p75. The binding activates at least three signal transduction pathways, the ras-erk pathway, the PI-3 kinase pathway, and a pathway terminating in the phosphorylation of a protein called SNT. These, in turn, lead to alterations in the phosphorylation and, consequently, the function of key proteins in the cell and to changes in the expression of specific genes. These changes in protein function and in gene expression, caused by the changes in phosphorylation, are the mechanism by which NGF exerts its effects on its target cells. Much of the work leading to this concept has been done with the PC12 pheochromocytoma, a cell line derived from a tumor of the rat adrenal medulla. This clonal line continues to be one of the most informative tools available for the study of NGF. In the presence of NGF, PC12 cells stop dividing, elaborate neurites, become excitable, and will synapse with appropriate muscle cells in culture. Indeed, they change from a rapidly-dividing chromaffin cell to a terminally-differentiated sympathetic neuron within a few days. The changes in phosphorylation that underlie these striking alterations in phenotype occur in virtually every compartment in the cell. Phosphorylation of NGF-stimulated calcium channels appears to regulate the calcium flux across the cell membrane and, in turn, the intracellular calcium levels, and these levels influence the survival of target neurons, regulate the ability of the neuron to withstand environmental insults such as stroke, and play a part in short-term synaptic plasticity. NGF-induced phosphorylation of the elements that control protein synthesis, such as eIF-4E, eEF-2, and S6, almost certainly alters the rate and the specificity of translation. The NGF-induced phosphorylation of certain transcription factors, such as NGFI-B, determines which genes are expressed at specific developmental stages and under specific physiological conditions. An understanding of the action of NGF will surely illuminate the control of neuronal differentiation and survival. Clinical interest in these peptide factors is intense because of the role they might play in neurodegenerative diseases.

Others:

G. Dickens	Biological Laboratory Technician	OSD, NICHD
M. Shibutani	Visiting Fellow	OSD, NICHD
Y. Katagiri	Visiting Fellow	OSD, NICHD
E. Kazanova (0.5 yr)	Visiting Fellow	OSD, NICHD
W. Zheng (0.25 yr)	Visiting Fellow	OSD, NICHD
M. Whalin	IRTA	OSD, NICHD
M. Monshipouri	IRTA	OSD, NICHD
H. Jiang	IRTA	OSD, NICHD
M. Czar (0.5 yr)	IRTA	OSD, NICHD
V. Movsesyan	Exchange Scientist	OSD, NICHD
P. Lazarovici	Exchange Scientist	OSD, NICHD
A. Chabuk (0.25 yr)	Student IRTA	OSD, NICHD
D. St. Ulme (0.5 yr)	Howard Hughes (High School) Intern	OSD, NICHD
M. Fasler (0.75 yr)	Adjunct Technician	OSD, NICHD

Cooperating Units:

Department of Pathology, Washington University School of Medicine
(J. Milbrandt)
Department of Cellular and Molecular Physiology, Harvard Medical School
(J. Blenis)
Department of Internal Medicine, Fukui University, Japan
(T. Mutoh)
Biomimetic Control Research Center, Nagoya, Japan
(Y. Hirata)
Department of Neurobiology, Weizmann Institute, Rehovot, Israel
(E. Yavin)
Department of Anatomy and Cell Biology, Georgetown University
(E. Yakovchenko)
Laboratory of Cell Biology, Food and Drug Administration
(D. Fink)
Laboratory of Chemical Physics, NIDDK
(I. Levin)

Project Description

Objectives:

The purpose of this project is to provide an understanding of the biochemical, molecular, and cellular actions of nerve growth factor (NGF). Such an understanding is important for a number of reasons. First, the action of nerve growth factor causes the survival and differentiation of many of its targets and information on how it works may shed light on the mechanisms of normal and abnormal neuronal development and differentiation and, perhaps, on the outlines of cellular differentiation in general. Second, nerve growth factor is the lead member of a class of homologous factors called neurotrophins and is a prototype for any number of related growth factors acting on neural tissue, and an understanding of the actions of nerve growth factor may point the way to an understanding of the actions of these other factors. Third, an understanding of the long-term actions of nerve growth factor in promoting the survival of certain of its target neurons may provide information of clinical value in cases where neurons die prematurely, such as in Alzheimer's Disease and Amyotrophic Lateral Sclerosis, or where neurons are damaged by their environment, such as in cases of stroke or peripheral neuropathies. Fourth, it has been recognized quite recently that the neurotrophins are involved in those short-term changes in neuronal plasticity that appear to determine appropriate connectivity in the nervous system and an understanding of its role may, indeed, lead to new information about the processes of learning and memory. Finally, since the actions of nerve growth factor on many of its targets are to decrease cell division, an understanding of the mechanism by which it acts may provide clues as to how the alternate cellular fates, differentiation and division, are controlled, not only in normal cells, but in the several tumor cells on which nerve growth factor acts. Interest in this field has increased dramatically over the last few years, due to a number of observations. First, the finding that nerve growth factor had actions in the central nervous system indicated that its role was not limited to specific portions of the peripheral nervous system. Then, the discovery of other neurotrophins broadened the field to include virtually all areas of the nervous system and to increasing aspects of normal development as well. Finally, the realization that these peptides could have clinical significance, in both neurological diseases and brain injury, has attracted significant commercial activity.

The approach taken in this laboratory has a number of separate, but related foci. First, we are interested in the reactions immediately following the combination of nerve growth factor with its receptors. These experiments involve an identification of the second messengers generated by such a combination and an exploration of the biochemical or biophysical reactions that control their levels. Second, we are concerned with the effect of these receptor-level events on protein phosphorylation reactions in the cell. Here we attempt to construct and dissect cell-free systems that reflect the actions of nerve growth factor on the intact cell. Third, we are involved in studies that explore the changes in the expression of specific genes responsible for the nerve growth factor-induced alterations in the properties of the cells, and how these gene-level changes are regulated. In these experiments we make use of molecular biological manipulations to alter the action of the trans-acting factors that control the expression of specific genes and to determine the effect of these alterations on the actions of nerve growth factor. Finally, we are attempting to understand how these biochemical and molecular changes alter the phenotype of the cell. In these studies we try to determine such things as the role of nerve growth factor-induced calcium uptake in the survival of the cell or the role of nerve growth factor-induced decrease in mitogen receptors in the control of cell division and neuronal maturation. The overall aim is to understand how nerve growth factor acts in biochemical, molecular, cellular, and clinical terms.

Methods Employed:

The methods used include the standard techniques of cell culture and analysis, almost exclusively with the nerve growth factor-responsive cell line PC12, its variants, and PC12 cells engineered to carry specific mutations. 3T3 cells and certain somatic cell hybrids are also used. Cell fractionation and enzyme assay are done routinely. Enzyme purification relies heavily on the use of fast protein liquid chromatography (FPLC). Protein phosphorylations are analyzed by polyacrylamide gel electrophoresis, either in SDS or acid-urea buffer systems, and phosphopeptide mapping is carried out by two-dimensional analysis or by HPLC. Specific kinases are investigated through the use of in-gel assays, in which the kinase-containing protein fractions are separated on SDS gels containing kinase substrates and then renatured in the presence of radioactive ATP to detect kinase activity. Peptide sequencing has permitted the identification of proteins, the phosphorylation of which is altered in nerve growth factor-treated cells. Polyclonal antibodies are prepared against proteins purified in the laboratory or against peptide sequences of interest prepared synthetically. These antibodies are also used to detect, by western blotting, or to isolate, by immunoprecipitation, proteins of interest. Commercial antibodies are frequently used immunohistochemically to localize specific proteins in or on cells. Various proteins are radioiodinated in order to measure the binding of these ligands to their specific receptors on the cell surface. Antibodies or specific toxins are introduced into cells using electroporation, or by permeabilization of the cells with mild detergents. The uptake of radioactive calcium into PC12 cells and the binding of radioactive ligands have been used to explore the role of specific calcium channels in the actions of nerve growth factor. Various toxins are evaluated for their effects on nerve growth factor-activated ion channels. Fluorescent intracellular calcium-binding dyes have been most valuable in the evaluation, by fluorescence-activated cell analysis, of the effects of growth factors on channel function. Various combinations of transfection or infection are employed to introduce specific genetic elements into the cells in order to evaluate their roles in the differentiation produced by nerve growth factor. The use of antisense probes has allowed the deletion of selected proteins and the construction of dominant-negative transfectants has permitted an analysis of the role of certain signal transduction molecules in nerve growth factor action. Site-directed mutagenesis is now employed to alter specific residues in the nerve growth factor receptors in order to determine the roles of these residues in signaling. Nucleic acid hybridizations have been of great value in studying, on northern blots, the patterns of gene expression following treatment of the cells with different growth and differentiation regulators. The translation of specific messages using a fortified rabbit reticulocyte system or an S10 extract of PC12 cells is now used to explore the regulation of translation during neuronal differentiation.

Major findings:

Our effort to elucidate the molecular events mediating the actions of nerve growth factor on its target cells has led to the following specific advances during the past year.

1) Calcium uptake into PC12 cells is increased by treatment of the cells with nerve growth factor. Furthermore, this action of nerve growth factor depends upon a unique calcium channel and the stimulating action of nerve growth factor on this channel is probably due to a phosphorylation of one or more of the channel subunits. It has been suggested that, since the survival of neurons requires adequate intracellular calcium levels, this action may be one of the mechanisms by which nerve growth factor keeps neurons alive. Present work focusses on the pathway by which this channel is activated and recent data implicate protein kinase C. Long-term down-regulation of kinase C with the tumor promoter PMA prevents the action of nerve growth factor on calcium uptake, and the use of isoform-specific effectors has identified kinase Cα as the kinase isoform involved in nerve growth factor action. The action of the kinase inhibitor, K-252a, previously shown also to stimulate calcium uptake into PC12 cells, is also prevented by down-regulation of kinase C and the same isoform-specific effectors that influence nerve growth factor action also influence the action of K-252a, suggesting that these agents act through one or more common cellular elements, perhaps the nerve growth factor receptor itself. Related studies with 3T3 cells transfected with nerve growth factor receptors show that both the high-affinity nerve growth factor receptor, $p140^{trk}$, and the low affinity nerve growth factor receptor, $p75^{NGFR}$, stimulate calcium uptake into PC12 cells, but perhaps by different mechanisms. This work highlights the role of calcium in the action of nerve growth factor and, in turn, the role of nerve growth factor in neuronal survival, neuronal protection, and neuronal plasticity.

2) A continuing interest in the nerve growth factor-stimulated phosphorylation of the ribosomal protein S6 has led, in previous work, to the identification of a unique 47 kDa nerve growth factor-stimulated S6 kinase in the cytoplasm of PC12 cells. It now appears that this new kinase is related immunologically to the $p70^{S6k}$ present in many cells. Not only do antibodies directed against $p70^{S6k}$ recognize the 47 kDa enzyme, but rapamycin, an inhibitor shown to inhibit the growth factor-induced activation of $p70^{S6k}$ in whole cells, inhibits the nerve growth factor-induced activation of the 47 kDa enzyme in PC12. Another interesting development has been the finding that the 47 kDa S6 kinase is rather specifically bound to ribosomes. Indeed, when compared to the other enzymes known to phosphorylate S6 in the cell, the 47 kDa kinase is bound in much greater proportion than the others. Although it is clear that in order to phosphorylate a ribosomal protein, these kinases must bind to the ribosomes at least transiently, the observation of the comparatively tighter binding of the 70 kDa kinase opens the door to studies of the specific binding sites on both kinase and ribosome. The study of the S6 kinases in PC12 cells has important implications for the action of nerve growth factor on protein synthesis and, in turn, on the phenotypic changes nerve growth factor induces in its target cells.

3) The transcription factor NGFI-B is induced by a number of different effectors in PC12 cells. Nerve

growth factor is among these, but nerve growth factor has the added effect of inducing a robust phosphorylation of the molecule. Previous work from this laboratory has shown that one specific nerve growth factor-induced phosphorylation has the effect of inhibiting the binding of NGFI-B to its target sequence on DNA and, presumably, limiting its transcriptional activity. A kinase, termed NGFI-B kinase I has been purified and characterized and it has been shown to phosphorylate a specific serine in the DNA-binding domain of NGFI-B. The identity of this kinase is not known for sure, but it is very similar or even identical to CREB kinase, the enzyme phosphorylating the protein that binds to the cAMP response element. In order to understand the biology of this apparently anomalous action of NGFI-B kinase, e.g., an enzyme induced by nerve growth factor that blunts the action of a transcription factor also induced by nerve growth factor, experiments have been done with other effectors that induce NGFI-B, but do not induce NGFI-B phosphorylation. It has now been shown that the induction of NGFI-B by depolarizing concentrations of K^+ causes increased transcription of a reporter gene linked to the NGFI-B response element. However, the addition of nerve growth factor as well inhibits this action of K^+. The data confirm the in vivo role of NGFI-B kinase, a role hitherto shown only in a cell-free system. They also highlight the importance of the NGF-dependent induction of NGFI-B kinase rather than of NGFI-B itself. Perhaps most important, they provide an example of the interaction of nerve growth factor with neuronal activity in the expression of specific genes. One interpretation of the data could be that at a certain developmental stage, perhaps upon synapse formation, the role of neuronal activity in gene expression is altered by the availability of agents such as nerve growth factor.

4) The finding, some years ago, that PC12 cells respond both to the differentiating agent nerve growth factor and the mitogen epidermal growth factor, has led to important studies on comparative signal transduction and to equally significant experiments on receptor interplay. The latter are based on the finding that differentiation with nerve growth factor causes the down-regulation of the epidermal growth factor receptor. Recent data have shown that mRNA levels for this receptor are unchanged even though receptor levels are markedly decreased. For this and other reasons, present work focusses on a possible nerve growth factor-induced decrease in the translation of the epidermal growth factor receptor mRNA. Two different translation systems are being used, one a fortified rabbit reticulocyte lysate and the other an S10 system from the PC12 cells themselves. Using these systems it has been possible to translate epidermal growth factor receptor mRNA. Over the past year constructs have been made that contain several hundred bases of the sequences flanking the epidermal growth factor receptor message linked to the reporter gene luciferase. These constructs are now being transcribed and translated in cell-free systems from naive and nerve growth factor-treated PC12 cells in order to determine, first, the influences that regulate epidermal growth factor receptor translation and, second, the involvement of nerve growth factor in that regulation. In a related line of research, the signal transduction pathway controlling this nerve growth factor-induced down-regulation is being sought. Using PC12 cells transfected with dominant negative signaling elements it has been shown that the ras-erk pathway is responsible for this down-regulation as it is for neurite outgrowth itself. However, it is clear that epidermal growth factor regulation is not linked to neurite outgrowth, as it occurs in nerve growth factor-treated cells that are prevented from attaching to a matrix, and it does not occur in cells treated, for example, with staurosporine, which make neurites but, as shown by recent data from this laboratory, by a different signal-transduction pathway than that serving nerve growth factor-induced neurite formation. These studies give promise of elucidating the molecular mechanism by which the levels of epidermal growth factor receptor are controlled during nerve growth factor-induced differentiation. Parallel studies suggest that both erbB2 and erbB3, receptors that are homologous with the epidermal growth factor receptor and are of significant clinical interest because of their possible role in the initiation or promotion of tumor growth, undergo similar nerve growth factor-induced down-regulation and that their levels may be controlled by mechanisms similar to those regulating the levels of the epidermal growth factor receptor.

Overall, research conducted under this project has shown that the nerve growth factor-induced phosphorylation of key proteins in nerve growth factor-sensitive cells is a major part of the mechanism by which nerve growth factor acts. These changes in phosphorylation and the consequent changes in function are the sum and substance of the phenotypic changes that nerve growth factor produces. Our recent studies have identified specific gene products, e.g., the nerve growth factor-sensitive calcium channel, protein kinase C, the ribosomal protein S6, the nerve growth factor-sensitive transcription factor NGFI-B, the epidermal growth factor receptor, that play crucial roles in these alterations in phenotype. Further, some of the newer experiments are designed to explore how these specific gene products contribute to the global changes in phenotype the cells experience under the influence of nerve growth factor.

Significance to Biomedical Research and the Program of the Institute:

The normal development of the nervous system is both intricate and precise and is integrated in both time and space. The appropriate neural connections must be made in exactly the right place and at exactly the right developmental times. Neurons reach their appropriate locations, stop dividing, and differentiate on an exquisitely complex and exact timetable and redundancies are eliminated by programmed cell death. It has become clear that the neurotrophins and other neural growth factors are key players in the control of these processes. Through chemotactic mechanisms they encourage the correct connections; through control of synaptic plasticity they provide for the adjustments in synaptic strength that appear to underlie brain function; through differentiative processes they inhibit cell division; through competition for their limited quantities they elicit programmed cell death. An understanding of the detailed mechanisms of these interactions will elucidate a number of fundamental biological processes. Such an understanding, also, will permit insights into what happens when these processes misfire. These insights should allow the formulation of clinical approaches toward the correction of these aberrant states. Through an understanding of such fundamental biological phenomena as process formation, synaptic plasticity, chemotactic attractions, and programmed cell death it should be possible to consider solutions to complex problems such as mental retardation. By exploring the control of normal neural cell death during development, information should be developed that is relevant to conditions involving abnormal cell death, such as Alzheimer's Disease, peripheral neuropathies, and stroke. The clinical use of growth factors in such conditions is a present reality and, since the rate of discovery of new growth factors is on an ascending curve, it is also an abundant promise for the future.

Proposed Course:

We will continue to explore the events immediately following the binding of nerve growth factor to its receptor, the pathways by which the signal generated at the receptor is transmitted throughout the cell, the changes these signals produce in the nucleus, and the role of specific gene products in the overall phenotypic changes that nerve growth factor produces. We will try to (1) determine the mechanism of the nerve growth factor-induced activation of protein kinase Cα; (2) elucidate the mechanisms by which the two different nerve growth factor receptors facilitate the uptake of calcium; (3) explore the specificity of the binding of the 47 kDa S6 kinase to the ribosomes and describe the binding sites on both enzyme and ribosome; (4) purify and characterize the unique nerve growth factor-activated 47 kDa S6 kinase from PC12 cells, understand the structural relationship of this enzyme to $p70^{S6k}$, and determine the sites at which it phosphorylates S6; (5) translate clones of the epidermal growth factor receptor that contain both 5' and 3' flanking information in the S10 fractions from control PC12 cells and from PC12 cells differentiated with nerve growth factor; (6) construct clones containing the flanking sequences of erbB2 and erbB3 for linkage to the luciferase message and eventual translation in the S10 system from PC12 cells; (7) elucidate the effect of the site-directed mutagenesis of specific amino acids in the transmembrane regions of the nerve growth factor receptor $p140^{trk}$ on the ability of nerve growth factor to act on the cells; (8) seek specific gene products whose expression is inhibited by the action of nerve growth factor on chronically depolarized PC12 cells and ask if these changes are the result of the phosphorylation of NGFI-B; (9) determine the mechanism of the down-regulation of the low-affinity nerve growth factor receptor by dexamethasone and its reversal by retinoic acid and explore whether this down-regulation is involved in cell survival; (10) exploit the nerve growth factor-induced decrease in cyclin F levels to determine the effect of the overexpression of cyclin F on the nerve growth factor-induced changes in the cell cycle.

Publications:

Hirata Y, Whalin M, Ginty DD, Xing J, Greenberg ME, Milbrandt J, Guroff G. Induction of a nerve growth factor-sensitive kinase that phosphorylates the DNA-binding domain of the orphan nuclear receptor NGFI-B. J Neurochem 1995;65:1780-1788.

Lazarovici P, Matsuda Y, Kaplan D, Guroff G. The protein kinase inhibitors K-252a and staurosporine as modifiers of neurotrophin receptor signal transduction. In: Lazarovici P, Gutman Y, eds. Signal transduction. Harwood Academic Publishers, gmbh, Amsterdam (in press).

Movsesyan V, Whalin M, Shibutani M, Katagiri Y, Braude E, Guroff G. Down-regulation of cyclin F levels during nerve growth factor-induced differentiation of PC12 cells. Exp Cell Res (in press).

Oshima M, Hirata Y, Guroff G. The nerve growth factor family. In: LeRoith D, Bondy C, eds. Growth factors and cytokines in health and disease. Greenwich, CT, Jai Press, Inc (in press).

Shibutani M, Kim E, Lazarovici P, Oshima M, Guroff G. Preparation of a cell-free translation system from PC12 cells. Neurochem Res 1996;21:801-807.

Tang T, Hirata Y, Whalin M, Guroff G. Nerve growth factor-stimulated nuclear S6 kinase in PC12 cells. J Neurochem 1996;66:1198-1206.

Whalin M, Guroff G. The use of rapamycin and wortmannin and the dissection of the signal transduction pathways regulating the phosphorylation of the ribosomal protein S6. In: Lazarovici P, Gutman Y, eds. Signal transduction. Harwood Academic Publishers, gmbh, Amsterdam (in press).

Yakovchenko E, Whalin M, Movsesyan V, Guroff G. Insulin-like growth factor-I receptor expression and function in nerve growth factor-differentiated PC12 cells. J Neurochem 1996;66:1868-1875.

Zheng W, Fink DW Jr, Guroff G. The role of protein kinase Cα in nerve growth factor-induced arachidonic acid release from PC12 cells. J Neurochem 1996;66:1868-1875.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01500-14 OSD

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on DNA Replication, Repair, & Mutagenesis in Eukaryotic & Prokaryotic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: A.S. Levine, M.D., Head, SDRRM, OSD, NICHD
E. Chumakov, Ph.D. - IRTA Fellow
M. Gonzalez, Ph.D. - NRC Fellow
J. Hauser - Microbiologist
G. Kopsidas, Ph.D. - Exchange Scientist
E. Maury - Tech IRTA Fellow
J. McDonald, Ph.D. - IRTA Fellow
M. McLenigan - Chemist
V. Rapic Otrin, Ph.D. - IRTA Fellow
O. Studitskaia, Ph.D. - Visiting Fellow
D. Vandewiele, Ph.D. - Visiting Fellow
K. Winterling - Pre-IRTA Fellow
R. Woodgate, Ph.D. - Visiting Scientist

COOPERATING UNITS *(if any)*

NLM (E. Koonin & J. Wootton); NIDDK (E. Ackerman); FDA (W. Koch); Columbia University, New York (T. Peat & W. Hendrickson); ICRF, London (R. Wood); University of Southern California (M. Goodman); University of Rochester (C. Lawrence); NIHS, Japan (T. Otha)

LAB/BRANCH

Office of the Scientific Director

SECTION

Section on DNA Replication, Repair, and Mutagenesis

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
12.0	9.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies on the mechanism of **mutagenesis** in **prokaryotic cells** focused on **RecA** and **UmuDC**-like mutagenesis proteins. We crystallized the UmuD′ protein; refined the structure to 2.5 Å; and elucidated the mechanism of self-cleavage that converts UmuD to the mutagenically active UmuD′. We also discovered that while UmuD′ forms a molecular homodimer, it can also form an extended polymeric structure that interacts with RecA* as well as UmuC, facilitating **translesion DNA synthesis** at the site of unrepaired DNA damage. We found that UmuD and UmuC proteins are inherently unstable in ***E. coli*** and are degraded by the ***Lon* protease**. UmuC is, however, protected when co-expressed with UmuD′. While the homodimeric UmuD′ protein is quite stable, it is rapidly degraded by the ***ClpXP* protease** when in a heterodimeric complex with UmuD. These interactions allow error-prone, Umu-mediated synthesis only as a last resort, avoiding gratuitous mutagenesis. Studies with ***X.laevis*** demonstrated that while oocytes can efficiently replicate undamaged ss DNA, they are unable to replicate DNA that contains adducts. This arrest was alleviated in progesterone-matured oocytes and in oocytes microinjected with umuDC mRNAs, suggesting that the basic mechanisms of mutagenesis are highly conserved. We analyzed the specificity of the mutator phenotype of a *recA730 lexA51*(Def) strain by employing F′-plasmids carrying a set of mutant *lacZ* genes that can individually detect specific transitions, transversions, and frameshift events. Most of the spontaneous mutagenesis could be attributed to a specific increase in A:T→T:A, A:T→C:G and G:C→T:A transversions. These events were completely abolished in a Δ*umuDC* strain, indicating that functionally active UmuD′C proteins are normally required for their generation. The spectrum obtained was similar to that of strains with a defect in the ϵ (3′-5′ proofreading) subunit of **DNA polymerase III**, raising the possibility that the wild-type ϵ protein is inactivated in strains expressing the RecA730 and UmuD′C proteins. We also used an ss DNA vector carrying a site-specific T-T cyclobutane dimer to analyze the mutagenic specificity of a set of isogenic *E. coli* Δ*umuDC* strains harboring low-copy-number plasmids expressing UmuD′C or its homologs (***mucA′B, rumA′B***). In *umuD′C* strains, 3′ T → A mutations outnumbered 3′ T → C, but the reverse was true for the homologs, suggesting that UmuD′C and its homologs may differ in their relative abilities to promote elongation from mismatched termini. To date, eight closely related homologs of the *E. coli* UmuC protein have been identified, with all appearing to play critical roles in damage-inducible mutagenesis in ***enterobacteriaceae***. A distantly related UmuC-homolog, ***DinB***, was also identified in *E. coli*, as was a new member of the UmuC-superfamily in the archeon, ***Sulfolobus solfataricus*** [***dbh*** (*dinB* homolog)].

In a second project, we studied a protein complex (**UV-DDB/XPE factor**) that binds to 6/4 photoproducts in UV-damaged human DNA. We found, in an *in vitro* nucleotide excision repair (NER) assay, that the addition of the complex leads to a modest stimulation of repair, suggesting that UV-DDB plays an accessory, but not a core role, in the NER process. We also found that the complex moves to a tight association with damaged DNA upon UV treatment of cells. **RPA**, which also redistributes after UV, is also present in the UV-DDB/DNA complex, and the interaction of DDB and RPA enhances the DNA binding of either alone, suggesting that UV-DDB functions in the initial recognition step of DNA repair.

PROJECT DESCRIPTION

OBJECTIVES:

A. *PROKARYOTIC MUTAGENESIS*

Under optimal conditions the fidelity of DNA replication in *Escherichia coli* is extremely high; on average, only one error occurs in every 10^{10} bases replicated. Upon exposure to a variety of DNA damaging agents, however, many *E. coli* strains exhibit a dramatic increase in the mutation frequency. Genetic and physiological experiments have indicated that DNA polymerase III holoenzyme (the main replicative enzyme of *E. coli*), RecA, and the UmuDC-like mutagenesis proteins are directly required for this process, all of which are induced as part of the cell's RecA-LexA controlled multi-gene SOS response to DNA damage. During the mutagenic process, RecA mediates the posttranslational cleavage of the UmuD-like proteins, leading to their activation for mutagenesis functions. The actual mechanism by which the UmuD'C-like and RecA proteins promote mutagenesis remains to be elucidated, although it is believed that they act together to promote translesion synthesis by DNA polymerase III holoenzyme. Thus, our continuing biochemical and genetic studies of the UmuDC-like proteins will provide us with a better understanding of the mechanisms of mutagenesis in *E.coli*, and in all likelihood, higher organisms.

B. *DNA REPAIR*

This project focuses on error-free DNA repair in mammalian cells, especially the initial step of the nucleotide excision repair pathway during which DNA damage is first recognized. We, and others, identified a protein that is induced, by DNA damage, to bind to the site(s) of damage in primate cells. In the system under study, damage is induced by UV light, and the UV-DNA damage binding (UV-DDB) protein binds selectively and specifically to one type of UV damage, 6-4 photoproducts – the most prevalent lesion induced by UV in transcribed DNA. We have found that in patients with one of the xeroderma pigmentosum variants, XP-E, UV-DDB binding activity is not inducible in some patients. In others, the DNA binding activity is present, and the phenotype (moderate skin sensitivity to UV) may be due to defects in the DDB protein that inhibit necessary protein-protein interactions. In addition to this somewhat ambiguous relationship of UV-DDB activity to the XPE phenotype, the DDB protein is not essential in an *in vitro* reconstituted nucleotide excision repair (NER) system, in contrast to all of the other known XP factors, suggesting that at least in NER, DDB may play an auxiliary rather than an essential role. Our current objective is to gain further insight into the precise role of the UV-DDB protein in NER, and to learn whether this complex might have additional roles in other macromolecular pathways in mammalian cells.

METHODS EMPLOYED

A. *PROKARYOTIC MUTAGENESIS*

Standard methods of molecular biology, biochemistry, and molecular genetics are employed in this work. Recombinant DNA clones have been constructed for the overproduction and characterization of the UmuD'-like and UmuC-like mutagenesis proteins. Plasmid DNAs are typically isolated using Qiagen columns and analyzed further by restriction enzyme digestion. Recombinant clones of interest are amplified by transformation of competent bacteria. Overproduced proteins are typically purified using ion-exchange, gel-filtration and affinity chromatography. Proteins are separated and analyzed using SDS-PAGE. Immuno-detection of proteins is achieved using the Western blotting technique, with visualization of proteins using a chemiluminescent substrate. Protein-protein interactions are studied directly using affinity chromatography and chemical cross-linking, or indirectly using the yeast "two-hybrid" system. Protein-DNA interactions are studied using an agarose based DNA mobility shift assay as well as filter binding assays. Novel bacterial strains used in this study are constructed using generalized phage P1 transduction.

B. *DNA REPAIR*

Standard methods of molecular biology and biochemistry are employed in this work, in addition to the use of various *in vitro* DNA repair assays. Standard methods of cell culture and cell fractionation are used to prepare whole-cell, nuclear, and cytoplasmic extracts. Liquid and affinity chromatography, and SDS-PAGE are used to purify DDB proteins. A damaged-DNA band-shift assay is employed to detect and characterize DDB proteins. DNA-binding substrates, specific DNA-cleavage reactions and protein fractions are analyzed by PAGE methods. PCR technology is applied to isolate cDNAs encoding DDB proteins. Standard methods of molecular biology are used to clone and sequence cDNAs. Cell transfection techniques are utilized for *in vivo* DNA repair studies and selection of cells carrying cloned DDB genes. Immuno-detection by Western blotting, and *in situ* by EM-immunochemistry, is employed to identify and localize DDB proteins, to quantitate DDB proteins in various subcellular fractions, and to identify proteins that interact with DDB. Enzyme and protein assays are carried out by standard biochemical techniques. DDB functions are assessed in an *in vitro* reconstituted NER system (R. Wood).

MAJOR FINDINGS

A. *PROKARYOTIC MUTAGENESIS*

Crystallographic studies of the *E. coli* UmuD′ protein

In an attempt to increase our understanding of the mutagenic process, we sought to determine the molecular structure of the mutagenically active UmuD′ protein. The protein was crystallized by the hanging drop method with a final protein concentration of 12 to 15 mg/ml. The protein adopts an apparently novel fold in that it is a tight beta structure with an extended amino terminus that has a single alpha helix encompassing residues 39 through 45. One turn, residues 61 though 64, adopts a 3_{10} helix conformation, and the rest of the structure is best described by seven beta strands connected by short loops. The seven strands are oriented to form three beta sheets, one with four strands and two with three strands. These sheets form the main body of the protein, which has two separate hydrophobic cores. The last beta strand, B7, is quite long and pairs in two different beta sheet formations. This strand is also interesting in that it slides between two other beta strands in the structure as described below.

The crystal is formed by two different kinds of protein interactions, one we call the molecular dimer and the other we call the extended or filament dimer. The molecular dimer interface is primarily hydrophobic and consists in part of residues val^{54}, ile^{87}, phe^{94} and phe^{128}, with the two phe^{94} residues, one from each monomer, being stacked on top of each other. There is also a salt bridge between the conserved residues lys^{55} and glu^{93} on both sides of the interface.

The filament dimer is formed by interactions at the amino and carboxyl termini of the protein. Residues 134 through 136 of the carboxyl terminus form hydrogen bonds across the interface with the related residues 134' through 136 '. This interaction extends two three-stranded beta sheets into a longer six-stranded beta sheet. A mutation at residue 138, methionine to either threonine or valine, is a gain of function mutant, showing higher levels of mutagenesis than the wild type protein. Although this residue is not clearly seen in the electron density maps, it is certainly in this interface. It seems likely that these mutants are forming better contacts either between the two UmuD' molecules or between the UmuD' and RecA proteins, thus forming a stronger filament.

Translesion DNA synthesis on *Xenopus laevis* oocytes

Closed circular ss DNA is efficiently replicated to ds DNA in *Xenopus* oocytes, eggs, and nuclear extracts. Oocytes do not replicate injected ds DNA, whereas *Xenopus* eggs and egg extracts replicate ~5% of injected ds DNA. Since oocytes and nuclear extracts are also both extraordinarily efficient at ds DNA repair, we investigated how oocytes and nuclear extracts might replicate/repair UV damaged ss DNA. ssDNA was irradiated with UV at various dosages injected into oocytes with a ^{32}P-labeled dCTP and analyzed. Virtually no DNA synthesis was observed on UV-irradiated circular ssDNA containing as few as ~2-3 cyclobutane dimers (CPD)/ss M13, while unirradiated ssDNA replicated to full length forms I and II.

To further characterize the replication arrest mechanism, we next determined if irradiated ss M13 prevents replication of unirradiated ssDNA, i.e., can the arrest inhibit all cellular DNA synthesis? Combining equal amounts of UV-irradiated ϕX174 with unirradiated M13, or vice-versa, only allows complete replication of the unirradiated DNA. Therefore, the oocyte replication arrest mechanism only applies to damaged template molecules. We also examined replication arrest in other *Xenopus* cells. Oocytes are arrested at first meiotic prophase; matured oocytes are released from this arrest, lack a nuclear membrane, and develop as far as metaphase of the second meiotic division. The same batch of oocytes unable to replicate irradiated ss M13 was able to replicate this substrate when it was injected after progesterone-induced maturation. Therefore, the replication arrest exhibited by oocytes with irradiated ssM13 is abrogated during meiosis. These results suggest the emergence in matured oocytes of a replicative bypass pathway. Alternatively, progesterone-induced maturation may effect a displacement of putative anti-replication protein(s).

Since the UmuD′C and MucA′B mutagenesis proteins are thought to act by facilitating translesion DNA synthesis in prokaryotic cells, we wanted to determine if they would perform similar roles in a heterologous system. Since producing functional UmuC or MucB has been intractable, the oocyte's translational proficiency was utilized by injecting mRNA transcripts encoding the prokaryotic proteins. Initially, the mRNAs were injected cytoplasmically, and after healing, the oocyte nuclei were injected with label and ss M13 DNA. Combinations of either *mucA′* + *mucB* or *umuD′* + *umuC* facilitated replication of the irradiated ss M13 template. mRNAs injected into the nucleus simultaneously with irradiated ss M13 also facilitated replication, consistent with reports that mRNAs injected into the nucleus are exported quickly to the cytoplasm. Only cognate mRNA combinations promoted significant replication. No replication occurred when non-cognate mRNA partners *mucA'*+ *umuD'*, or *umuC* + *mucB*, were injected and only limited synthesis was seen with *umuD'* + *mucB*. Thus, UmuD'C and MucA'B abrogate the replication arrest in oocytes and facilitate some complete bypass synthesis. We have initiated experiments to determine whether the replication observed is translesion and/or mutagenic.

Purification of soluble UmuD′C

A major goal of this study was to purify and characterize a complex containing UmuC and UmuD' (UmuD'C complex) while maintaining the complex in a soluble form throughout each purification step. Overproduction of UmuD'C in its native form was accomplished by placing the operon on a plasmid under ptac control. In the initial purification step, nucleic acids were precipitated by addition of polyethyleneimine. UmuC and UmuD' coprecipitated with the nucleic acid and were extracted in the presence of 1M salt and dialyzed into 50 mM NaCl. The remaining purification steps involved separation on DEAE-Sephacel, phosphocellulose, and Superdex-75 columns, followed by concentration using a Centricon 30 centrifugation cartridge. UmuD'C was present in 1 M NaCl following fractionation on Superdex-75. Attempts to reduce the salt concentration subsequent to gel filtration, e.g., by dialysis, were unsuccessful because of the formation of a heavy insoluble protein precipitate containing both UmuC and UmuD'.

The apparent molecular weight of the complex, determined by gel filtration on an analytical Sephadex G100 column in the presence of 1M NaCl, is approximately 70 kDa. An estimated molecular weight of 70 kDa is in agreement with the expected composition of a complex containing one UmuC (46 kDa) and two UmuD' (12 kDa) molecules. Analysis of the DNA binding properties of UmuD′C revealed that it does not bind to double-stranded DNA, but binds cooperatively to a single-stranded DNA. No detectable protein was bound at low UmuD'C/DNA ratios. In the "steep-binding" range, binding increased from about 1 UmuD'C bound per 300 nucleotides to about 1 per 50 nucleotides. Saturation of the ss 600 mer occurred when approximately one UmuD'C was bound per 20 nucleotides. We estimated a value of K_D = 260 nM for the apparent binding constant of the complex to the 600 mer.

Two experiments were performed to visualize the binding of UmuD'C to M13 single stranded DNA in the presence of activated RecA protein, preincubation of RecA with DNA followed by the addition of UmuD'C and preincubation of UmuD'C with DNA followed by addition of varying levels of RecA*. The UmuD'C-M13 DNA complex formed in the absence of RecA* caused an acceleration of the DNA. In contrast, however, when RecA was pre-incubated with DNA (in the presence of ATPγS) to form a RecA*-nucleoprotein filament and UmuD'C subsequently added to the reaction, a retarded complex was observed (Complex II) that is consistent with it being a UmuD'C-RecA*-DNA complex. Interestingly, under conditions where a significant fraction of the ssDNA would be expected to be bound by RecA*, there is a significant increase in the amount of free UmuD'C complex. This observation suggests that UmuD'C is unable to out-compete the RecA from the ssDNA once it has formed a RecA*-nucleoprotein filament.

Preincubation of UmuD'C with DNA, followed by the addition of three different levels of RecA, resulted in a series of retarded complexes that are consistent with them resulting from a UmuD'C-DNA complex together with a variable amount of RecA* protein, suggesting that prior binding of UmuD'C to DNA may inhibit the extent to which RecA can form a nucleoprotein filament.

Analysis of chimeric *Salmonella typhimurium* UmuC proteins

Unlike *Escherichia coli*, the closely related bacterium, *Salmonella typhimurium*, is much less responsive to the mutagenic effects of DNA damaging agents. Previous experiments reported by Sedgwick *et al.* (Mol. Gen. Genet. (1991) 229:428-436) have suggested that these phenotypic differences might result from a reduced activity of the *S. typhimurium* UmuC protein. To investigate this possibility, we have taken advantage of the high degree of homology between the *E. coli* and *S. typhimurium* UmuC proteins, and have constructed a series of plasmid-encoded chimeric proteins. The possibility that the phenotypic differences might be due to differential expression of the respective UmuC proteins was eliminated by constructing chimeric proteins that retained the first 25 N-terminal amino acids of either UmuC (and presumably the same translational signals), but substituting the remaining 397 C-terminal amino acids from the reciprocal operon. Constructs expressing mostly *E. coli* UmuC were moderately proficient for mutagenesis, whereas those expressing mostly *S. typhimurium* UmuC exhibited much lower mutation frequencies, indicating that the activity of the structural *S. typhimurium* UmuC protein is indeed curtailed. The regions responsible for this phenotype were more precisely localized by introducing smaller segments of the *S. typhimurium* UmuC protein into the *E. coli* UmuC protein. While some regions could be interchanged with few or no phenotypic effects, substitution of *E. coli* residues 212-395 and 396-422 with those from *S. typhimurium* resulted in reduced mutability, while substitution of residues 26-59 caused a dramatic loss of activity. We suggest, therefore, that the primary cause for the poor mutability of *S. typhimurium* can be attributed to mutations located within residues 26-59 of the *S. typhimurium* UmuC protein.

Factors affecting the *in vivo* stability of the *E. coli* Umu proteins

We have previously determined that when fully derepressed, wild-type cells contain approximately 2400 UmuD molecules and 200 UmuC molecules per cell. Certain *recA* mutants constitutive for its coprotease activity (*recA**) have 3-5 fold higher steady-state levels of the Umu proteins. We hypothesized that this increase might occur through a direct protein-protein interaction between the RecA* and Umu proteins that protects the Umu proteins from cellular degradation. To examine this possibility, we have constructed a novel *umuDC* mutation that substitutes the chromosomal *umuDC* genes with that of the *ermGT* gene encoding erythromycin resistance. The Δ(*umuDC*)*596*::*ermGT* allele was subsequently moved via generalized P1 transduction into *recA*$^+$ and *recA730* backgrounds. Low copy plasmids encoding UmuD, UmuD′ and UmuC were introduced into these two strains and the half-lives of the Umu protein analyzed. When expressed on their own, both the UmuD and UmuC are unstable. We were therefore interested in determining which of *E. coli*'s many proteases was responsible for their degradation. Clues to the identity of the protease came from observations that certain strains exhibiting extremely stable levels of UmuC also exhibited a mucoid phenotype. Such a phenotype is often associated with defects in the Lon protease because of an increase in capsule synthesis mediated by the positive regulator, RcsA. To test the hypothesis that Lon may be involved in the degradation of the Umu proteins, we compared the stability of each of the separately plasmid-encoded Umu proteins in isogenic Δ*umuDC lexA51*(Def) strains carrying either lon$^+$ or *lon*$^-$ alleles. Indeed, the presence of the *lon146*::Tn*10* mutation had a dramatic effect on the stability of both UmuD and UmuC proteins. This observation strongly suggests that the UmuD and UmuC proteins are substrates of the Lon protease. Compared to UmuD, UmuD′ is much more stable in the Δ*umuDC* background, and is relatively insensitive to a variety of proteases. Furthermore, we found that in the Δ*umuDC* background, UmuD′ interacts with UmuC in such a way as to form a very stable (and mutagenically active) UmuD′$_2$C complex that protects UmuC from degradation by the Lon protease. Given the tight regulation that *E. coli* appears to have evolved to keep the mutagenically active UmuD′C complex to a minimum, it seemed somewhat paradoxical that a cell would maintain high steady-state levels of the mutagenically active UmuD′C complex long after most DNA damage would be expected to have been repaired. We therefore hypothesized that there must be some mechanism available that would regulate the intracellular levels of the UmuD′C proteins. We considered the possibility that the formation of UmuD$^+$/UmuD′ heterodimers might provide such a regulatory function. In the experiments described previously, the UmuD′ protein was expressed in a Δ*umuDC* background and could only form homodimers. We were therefore interested in analyzing the stability of UmuD′ under conditions where it might be expected to be in a heterodimeric complex with UmuD rather than in a homodimer with itself. To do so, we employed the Δ*umuDC* strains harboring a low-copy-number plasmid, pRW366, that expresses UmuD′ and a non-cleavable UmuD1 protein *in cis*. (The use of a non-cleavable UmuD protein reduced the possibility that there may be some gratuitous generation of UmuD′ from UmuD). While the stability of the mutant UmuD1 protein was comparable to the wild-type protein, analysis revealed that in a wild-type background, steady-state levels of UmuD′ were, in stark contrast to the homodimeric UmuD′ protein, barely detectable. Similar results were also obtained when pRW366 was introduced into a *clpA319*::Kan and *clpB*::Kan strain. In contrast, when the very same plasmid was introduced into a *clpX*::Kan or *clpP*::Kan strain, UmuD′ was expressed and was apparently very stable. Together, these observations suggest that UmuD′ is only rapidly degraded by the ClpXP protease when it is complexed with UmuD.

Given the effects that Lon and ClpXP play in the degradation of the Umu proteins, we were interested in determining the UV-mutagenesis phenotype of *lon* and *clpXP* strains. Although the *lon* mutant exhibits greatly elevated steady-state levels of both UmuD and UmuC proteins, this does not seem to have a dramatic effect on UV-mutagenesis. In dramatic contrast, both the *clpX*::Kan and *clpP*::Kan strains were rendered phenotypically poorly-mutable by UV-light when compared to the isogenic *clp*$^+$ strain. Based upon our previous data, we expect that the chromosomally encoded UmuD$^+$ and UmuD′ proteins are more likely to exist as a heterodimer in these strains, and we believe that this observation is consistent with the hypothesis that heterodimers are mutagenically inactive.

Identification of a UmuC homolog in Archea.
One of our long term goals is to identify mutagenesis proteins in higher organisms. To date, molecular analysis has revealed true *umu*-like genes in a variety of gram-negative enteric bacteria. Based upon DNA hybridization studies, a *umu* homolog has been reported in the gram-positive *Streptomyces coelicolor*, but the structure of these *umu*-like genes still awaits identification. Considering their abundance, at least in gram-negative enteric bacteria, and their apparent conservation throughout evolution, it is possible that the mutagenesis proteins in higher organisms are structurally, as well as functionally, related to the prokaryotic family of Umu-like proteins. To identify these proteins we have utilized degenerate oligonucleotides to regions that are conserved in the previously identified UmuC-like proteins. Using this approach, we have identified a putative UmuC-like homolog in the Archeon *Sulfolobus solfataricus*. The fragment was cloned, sequenced, and found to share significant similarity with the *E. coli* UmuC and DinB proteins. This fragment was subsequently used as a probe to identify and clone the entire *dbh* gene from a *S. solfataricus* pBK-CMV phagemid library. Several positive clones were identified, and DNA sequence analysis of one of these clones, pOS21, revealed that the *dbh orf* consists of 351 codons and encodes a putative protein with an M_r of 40 kDa and a theoretical pI of 9.37. The encoded protein showed 39% identity with the *E. coli* DinB protein over a 218 amino acid residue overlap, and 29% identity with the *E. coli* UmuC protein over the same 218 amino acid stretch. The *orf*, located immediately upstream of the *S. solfataricus dbh* gene, encodes a putative protein that consists of 287 amino acid residues, and shows a significant similarity to ribokinases and related sugar kinases (26% identity with the *Bacillus subtilis* ribokinase). The termination codon of this gene, and the initiation codon of the *dbh* gene, overlap by four base pairs, suggesting that they may exist in an operon and might be translationally coupled. While in *E. coli*, both *umuC* and *dinB* are negatively regulated at the transcriptional level by the LexA repressor, and are induced as part of the global response to DNA damage – no sequence resembling the LexA binding site was observed upstream of either the *S. solfataricus dbh* gene or the putative ribokinase gene. Thus, at this time we have no indication that *S. solfataricus dbh* is damage-inducible. A partially sequenced *orf* further upstream of the ribokinase gene encodes the N-terminal portion of a protein that is closely related to two uncharacterized yeast proteins, YD9346.02c and SC38KCXVI_20 (more than 50% identity with each) and more distantly related to the RuvB subunit of the bacterial Holiday junction-specific helicase, RuvAB. The sequenced portion of the *S. solfataricus* protein includes the principal ATP-binding motif, and the protein is predicted to possess ATPase activity.

In order to better evaluate the relationships between different UmuC-like proteins, we undertook an iterative search of protein and nucleotide sequence databases with the sequences of all proteins known to belong to this superfamily. As a result, we detected six UmuC-related proteins that have not been described previously, namely a *C. elegans* protein most closely related to yeast REV1, two homologs from *Bacillus subtilis*, a homolog from *Mycobacterium tuberculosis,* as well as putative proteins from *Lactococcus lactis* and *Synechocystis sp.* with low similarity to the other members of the superfamily. In addition, two putative human ESTs related to the UmuC-like proteins were detected. The multiple alignment of the UmuC-like superfamily of proteins includes 5 distinct conserved motifs, with the most prominent conservation observed in motif III. Previously described *E. coli umuC* mutations are localized to these motifs, emphasizing their importance for protein function. However, the biochemical basis of this function remains unknown. Additional database searches using each of the conserved motifs to derive a position-dependent weight matrix failed to identify statistically significant matches to any other proteins. Nevertheless, it may be interesting to note that the most conserved portion of motif III, with adjacent invariant aspartic acid and glutamic acid residues preceded by a stretch of hydrophobic residues, resembled the Mg^{2+}-binding site that is conserved in a variety of ATPases. The high level of sequence conservation between UmuC-like proteins from bacteria, archea, and eukaryotes suggests that these proteins may have an enzymatic activity, the nature of which remains to be determined.

Spectra of Spontaneous and Damage-Induced mutations generated by the Umu, Muc and Rum proteins.

To date, eight *umu*-like operons have been cloned and sequenced (including the three *umu*-like genes that we have identified). Although these operons are structurally similar, the *umu*-like operons all differ in their ability to enhance cellular and bacteriophage mutagenesis.

Analysis of the spontaneous mutations recovered from *recA*$^+$ *lexA*$^+$ strains revealed that base-pair substitution transition and transversion events were very low (generally <1 Lac$^+$ revertants / 10^8 cells) while frameshift events exhibited relatively higher frequencies with 15-22 Lac$^+$ revertants / 10^8 cells. By comparison, analysis of the spontaneous mutations recovered from the *recA730 lexA51*(Def) strain indicated that the *recA730* mutation preferentially stimulated certain transversion events. In particular, the frequency of A:T->T:A, A:T->C:G and G:C->T:A were elevated. Compared to the corresponding wild-type (*recA*$^+$ *lexA*$^+$) strain, the mutation frequency of A:T->T:A transversions increased roughly 30-fold, and that of A:T->C:G and G:C->T:A transversions 23-fold and 18-fold respectively. In contrast, none of the transition or frameshift events examined here appeared to be significantly altered in the presence of the *recA730* mutation.

Since the spontaneous mutator activity of *recA** strains has previously been shown to be Umu-dependent, we were interested in determining whether the observed spectral changes were also dependent upon Umu activity. Analysis revealed that the Δ*umuDC* mutation completely abolished the mutator phenotype of the *recA730* strains. Furthermore, the levels of A:T->T:A, A:T->C:G, and G:C->T:A transversions in a *recA730 lexA51*(Def) Δ*umuDC* background were indistinguishable from their levels in *recA*$^+$ *lexA*$^+$, *recA*$^+$ *lexA51*(Def), or *recA*$^+$ *lexA51*(Def) Δ*umuDC* backgrounds. Interestingly, the *recA*$^+$ *lexA51*(Def) strain exhibited similar mutation frequencies to that of the *recA*$^+$ *lexA*$^+$ strain, indicating that simple derepression of the SOS regulon (including the Umu proteins) is not sufficient to promote the transversion events. In the *recA*$^+$ *lexA51*(Def) strain, virtually all of the UmuD protein remains in its unprocessed inactive form. In contrast, *recA730 lexA51*(Def) strains constitutively convert UmuD to the mutagenically active UmuD′ protein. Thus, it can be concluded that the stimulation of transversions observed in the *recA730 lexA51*(Def) background appear to be dependent upon the presence of the UmuD′C proteins.

MucAB and RumAB proteins are more effective than UmuDC at promoting replication past the T-T dimer, probably because both MucA and RumA are more susceptible to proteolytic processing and the processed proteins are better at interacting with limited amounts of activated RecA protein. These differences can be readily seen by comparing the efficiency of bypass promoted by the native wild-type operon and the genetically engineered active species in an undamaged cell. Under these conditions, UmuDC gave only 3% bypass, whereas UmuD'C resulted in a 14-fold higher figure with 42% bypass. In contrast, both MucAB and RumAB (which are efficiently processed and are likely to exist in their processed form even in an undamaged cell) gave much higher levels of bypass with 36% and 58% bypass, respectively. Introduction of the genetically engineered MucA'B and RumA'B resulted in a modest increase to 63% and 70% bypass, respectively. Interestingly, prior exposure of cells expressing MucA'B and RumA'B to UV-light failed to increase the extent of translesion DNA synthesis. These observations suggest that basal expression of the plasmid encoded MucA'B and RumA'B proteins is sufficient to promote bypass of a single lesion-containing molecule *in vivo*. It also suggests that the low level of activated RecA that is always present in *lexA*$^+$ cells is sufficient to target the MucA'B and RumA'B proteins to the lesion-containing DNA. The converse is equally true for UmuD'C. UV-irradiation enhanced bypass from 42% in undamaged cells to 70% in UV-irradiated cells. Thus, it seems likely that the differences observed between the *umu*, *muc* and *rum* operons occurs as a result of the relative proclivity of the UmuD-like protein to undergo posttranslational processing and its subsequent ability to interact with a RecA nucleoprotein filament. Under conditions where these steps are no longer limiting (i.e., in a UV-irradiated cell expressing the UmuD'C-like proteins) all three operons gave similarly high levels of bypass (~70%), indicating that the three mutagenic DNA repair complexes do not differ in an inherent ability to promote dimer bypass. Such a conclusion does not appear to apply to all mutagenic lesions, however. Data for abasic sites indicate that *mucAB* is probably intrinsically superior at bypassing this lesion. The results also show that the relative abilities of *umuDC*, *mucAB*, and *rumAB* to promote translesion synthesis vary according to the particular lesion concerned.

Analysis of the mutagenic spectra generated in the bypass reaction revealed that *mucAB* and *rumAB* change the relative frequencies of the two major types of mutations induced by the T-T dimer, but do not increase the overall error rate. In previous experiments with a chromosomal copy of *umuDC*, we have shown that replication past the T-T cis-syn cyclobutane dimer *in vivo* is largely accurate, with an error rate of only 6% - 7%. Two major classes of mutations are induced, both at the site of the 3' thymine, namely 3' T→A and 3' T→C , with relative frequencies of about 5:1. In the recent experiments, we found that the relative frequencies of these mutations vary according to the particular operon expressed. For example, 3' T→A mutations are recovered more frequently than 3' T→C mutations in cells expressing the UmuDC proteins. This is not the case in strains expressing the MucAB or RumAB proteins, however. In these, 3' T→C mutations are consistently recovered at a higher frequency than 3' T→A mutations. In most instances, the ratio appeared to be reversed and 3' T→C mutations outnumbered 3' T→A mutations by about 5:1. Finally, although the MucAB and RumAB proteins alter the ratio of the mutation frequencies, they do not increase the overall error rate. As judged by the results from the single T-T dimer, the enhanced frequencies of UV-induced mutations observed in strains expressing the *mucAB* or *rumAB* genes do not appear to result from any general increase in the error rate of translesion synthesis, but are more likely achieved by their superior abilities to promote translesion synthesis.

B. *DNA REPAIR*

DNA repair is an essential cellular process that preserves genomic integrity and programmed gene expression, and protects a cell from neoplastic transformation and death. Various DNA lesions, induced by endogenous or environmental agents, are efficiently recognized and repaired by different repair pathways in both prokaryotic and eukaryotic cells. The importance of the repair processes for human health has become apparent from studies on human hereditary diseases associated with a deficiency in DNA repair: e.g., a high susceptibility of xeroderma pigmentosum (XP) patients to sun-induced skin cancer is strongly correlated with the hypersensitivity of XP cells to UV light, and their decreased capacity to excise UV-induced photoproducts. Recently, several genes and gene products that are defective in XP cells have been isolated and cloned from normal mammalian cells. This small subset of about 25 gene products implicated in eukaryotic nucleotide excision repair (NER) has uncovered the complexity of the DNA repair apparatus but provided only a partial understanding of mammalian NER processes. In our studies, we have focused on the early step of NER – lesion recognition – with the goal of identifying proteins that interact with damaged DNA and which may form a repair-initiation complex.

In vitro and *in vivo* functional studies of purified UV-DDB

In collaborative studies with Dr. Rick Wood, ICRF, we found that a highly purified UV-DDB protein complex is able to replace one of the nuclear extract fractions containing this complex, which Wood et al. had previously used in an *in vitro* nucleotide excision repair (NER) reconstituted system. Addition of the purified protein complex to the *in vitro* system led to a two-fold stimulation of repair, suggesting, as in previous studies with a less purified fraction, that the UV-DDB complex plays an accessory but not a core role in the NER process. To elucidate further the role of the UV-DDB protein complex in NER, we have purified this protein to a very high degree of homogeneity by passing nuclear extract from HeLa cells through three UV-DNA cellulose columns with increasing salt concentrations in a binding buffer. Silver staining of this purified fraction revealed the major band to be p127, but the second known component of the UV-DDB complex, p48, was not detectable although this purified fraction retains high binding activity on DNA damaged probes (work in other laboratories has suggested that p48 is the subunit that directly binds to DNA). Experiments are currently in progress to test whether our purified DDB complex can restore wild-type NER DNA synthesis in fibroblasts from XPE patients that are known to be defective in the UV-DDB binding activity (both *in vivo* and *in vitro*), and hopefully, to finally clarify the question of whether the UV-DDB complex is indeed the XPE factor. Moreover, we have recently found that the UV-DDB protein complex comprises not only p127 and p48, but apparently, RPA as well. This association was earlier seen *in vivo*, and we now find that p127 and RPA form a specific association as detected by coimmunoprecipitation. p127 and RPA move from low-salt to high-salt chromatin after UV treatment of cells, indicating a translocation from low affinity to high affinity binding to chromatinized DNA. Finding that the UV-DDB complex moves to a tight association with damaged DNA upon UV treatment of cells, with the protein remaining on the DNA until the damage has been repaired, suggests, as noted above, a role in the initial recognition step of DNA repair. However, given the results in the *in vitro* NER reconstituted system, it appears that the UV-DDB complex plays only an accessory role in this recognition step. Since the XPA factor, another component of the human NER pathway (a defect in which leads to a severe phenotype), is known to recognize many types of DNA lesion, it is possible that UV-DDB can be replaced by XPA even if the former factor has greater affinity and specificity for 6-4 photoproducts. Since it is known that RPA also associates with XPA, that RPA also redistributes after UV, and that RPA is present in the UV-DDB/DNA complex, the suggestion once again is that UV-DDB functions in the initial recognition step of DNA repair. Interestingly, the interaction of DDB and RPA enhances the DNA binding of either alone, further supporting this notion.

Characterization of p48

As noted above, the UV-DDB protein complex has been purified from primate cells as a heterodimer of the 127 kDa protein subunit and a 48 kDa protein subunit (homodimers of 127 kDa subunits are found as well, and in fact, more commonly). Although the weight of the evidence suggests that p127 is the actual DNA binding protein, the function of p48 in the formation of the binding complex remains unresolved, as does the precise role of RPA. To add to the confusion, recent work in other laboratories has failed to demonstrate a clear association between apparent mutations in the genes encoding any of these subunits and the XPE phenotype. To further address these issues, we are attempting to produce specific antibodies to p48 (antibodies to p127 and RPA are already in hand). We have recently overproduced and purified p48 using a bacterial expression construct, making this goal feasible. In the next year, we shall focus on determining the precise roles of p127, p48, and/or RPA in both the initial and later steps of NER, using the *in vitro* system, defined DNA-lesioned probes, and purified DDB complex proteins known to reverse the XPE defect *in vivo*.

SIGNIFICANCE

A. *PROKARYOTIC MUTAGENESIS*

The products of the *umuDC* genes are required for most inducible mutagenesis in *Escherichia coli*. Our studies indicate that related proteins are required for similar processes in other enterobacteria and even more evolutionarily evolved organisms. The biochemical role of these so-called mutagenesis proteins still remains to be elucidated. In an effort to address these problems, the crystal structure of the *Escherichia coli* UmuD′ mutagenesis protein was determined at 2.5Å resolution. The structure provides the first insights into the mechanism of the RecA-mediated self-cleavage reaction that this protein, and a host of homologous proteins, undergo upon induction of the SOS response. Analysis revealed that the structure is a very compact beta fold. Based upon the structure, we have hypothesized that, in addition to forming a "molecular" dimer with itself, UmuD′ can form an extended "polymer" structure that is probably critical to its role in damage-induced mutagenesis.

Our studies with *Xenopus* oocytes and oocyte nuclear extracts revealed that while they are extremely efficient at both the repair of UV-photoproducts on double-stranded (ds) DNA and the replication of single-stranded (ss) to (ds) DNA, they are normally unable to replicate UV-irradiated ssM13 DNA containing cyclobutane pyrimidine dimers. The arrest was stage-specific and abrogated in progesterone-matured oocytes. Moreover, the replication arrest was alleviated by injection into oocytes of mRNAs encoding the UmuD'C or MucA'B mutagenesis proteins. Our results may explain how cells stabilize repair/replication events on DNA with unrepairable lesions, and avoid DNA damage-induced apoptosis once irreversibly committed to S-phase. The results also suggest that a eukaryotic mutagenic pathway only becomes apparent after meiosis, and appear to reflect a remarkable conservation of prokaryotic to vertebrate replication machinery.

Indeed, we have identified a potential UmuC-like protein in the archeon *Sulfolobus solfataricus*. While this is the first report of a UmuC-like homolog in an archeon, we detected additional homologs using protein sequence comparisons in Gram-positive bacteria, cyanobacteria, and among potential human EST products, indicating that UmuC-related proteins comprise a ubiquitous superfamily of proteins probably involved in DNA repair and mutagenesis.

Previous attempts to overproduce and purify UmuC required that insoluble UmuC protein be extracted from inclusion bodies, solubilized and purified in the presence of 8M urea, and subsequently refolded using either ribosomal protein or by sequential incubation with Hsp70 and Hsp60 heat shock chaperone complexes. The effects of denaturation and renaturation on the biological properties of UmuC are not known. Therefore, a major goal was to purify and characterize a complex containing UmuC and UmuD' (UmuD'C complex) while maintaining the complex in a soluble form throughout each purification step. Our initial biochemical characterization of the complex revealed that UmuD′C was unable to bind to dsDNA, but instead bound cooperatively to ssDNA. While much remains to be learned about SOS-dependent, spontaneous and damage-induced mutagenesis, a strategy to purify soluble UmuD′C proteins means that the prospects for elucidating the molecular mechanisms of these pathways is particularly encouraging.

Characterization of mutator phenotypes often illuminates various pathways of DNA repair and mutagenesis. The studies presented here show that the predominant mutational event recovered in a *recA730 lexA51*(Def) strain was an A:T->T:A transversion. While at the present time, the mechanisms by which the A:T->T:A transversions are produced is unknown, it has previously been shown that *mutD5* strains of *E. coli* (which carry dominant mutations in the *dnaQ* gene; ε proofreading subunit of DNA polymerase III) exhibit somewhat similar mutational spectra. Interestingly, two mutator alleles, *mutA* and *mutC*, have also been shown to produce the same general spectra. Very recently, these mutations have been localized to *glyV* (*mutA*) and *glyW* (*mutC*). Both alleles result in a tRNA that inserts glycine at aspartic acid codons, and it has been hypothesized that such events are likely to result in a defective ϵ subunit with properties similar to MutD5. The fact that the mutational spectra of the *recA730 lexA51*(Def) strain is similar to that of a strain with defective proofreading activity therefore raises the intriguing possibility that during SOS-dependent spontaneous mutagenesis, proofreading activity is temporarily inhibited. Such an hypothesis is consistent with previously published data, suggesting that ϵ is inactive during UV-induced mutagenesis and that RecA inhibits proofreading *in vitro*.

B. *DNA REPAIR*

In our studies on the mechanisms of DNA repair in mammalian cells, we have identified a DNA-binding activity, UV-DDB, which is specific for UV-induced 6-4 pyrimidine dimers. We purified a UV-DDB protein complex from primate cells and isolated a cDNA encoding a 127 kDa component of the ~210 kDa complex. p127 is a structurally novel, evolutionarily conserved member of a family of proteins that may be involved in the recognition step of DNA repair. Because the UV-DDB activity is defective in at least some patients with the XP group E syndrome, we may be able to ascribe a specific repair function to this DNA-binding complex and identify a molecular defect in XP-E patients. This is of great interest because a biochemical defect in this sun-sensitive, cancer-prone disorder has not yet been defined. In addition, our finding that some mammalian tissues (brain, ovaries) do not express UV-DDB activity, and therefore may have a slower rate of removal of DNA lesions, sheds new light on the importance of DNA repair in rapidly proliferating tissues (e.g., skin) versus tissues that do not proliferate (e.g., brain). Furthermore, by examining UV-DDB activity in other DNA repair disorders, as well as in aging and cellular transformation, we should be able to obtain a much broader view of the role of DNA repair in the important life processes. In addition to its importance for human health, our discovery of the structural similarity of p127 to yet uncharacterized proteins from slime mold, *Drosophila* and rice is of consequence to our understanding of the evolutionary appearance of this DNA-binding protein – with respect to both its function and structure. Site-directed mutagenesis studies of p127, as well as identification of putative mutations in UV-DDB mRNAs isolated from XP-E patients who lack UV-DDB activity, should allow the definition of a novel, yet unrecognized, DNA-binding motif in the UV-DDB protein which interacts specifically with UV-damaged DNA. Since the role of UV-DDB in DNA repair remains, however, not clearly defined, the ultimate significance of this protein may emerge in another cellular pathway entirely.

PROPOSED COURSE

A. *PROKARYOTIC MUTAGENESIS*

While solving the crystal structure of the UmuD′ protein has clearly provided great insights into the structure of the protein, much still remains to be determined. Perhaps the greatest interest is the role that the "polymer" structure plays in damage inducible mutagenesis. Using the calorimetric papillation assay that we have previously described and the yeast "two hybrid" system, we plan to identify mutants of *umuD′* that affect this interaction. Biochemical studies have previously shown that UmuD′ interacts with both RecA and UmuC. Determining these sites will provide us with a better understanding of how these proteins interact to form the so-called "mutasome".

Our finding that the prokaryotic mutagenesis proteins can function in the heterologous *Xenopus laevis* system, and thereby promote translesion DNA synthesis, is especially exciting. We plan to extend these studies by using UV-irradiated derivatives of M13mp2 that can be used to distinguish if the bypass synthesis is mutagenic based upon a simple calorimetric assay. Analysis of the mutagenic spectra obtained in the presence and absence of the micro-injected prokaryotic mutagenesis system, and comparison of the spectra with that found in the native prokaryotic environment, should provide additional evidence that the molecular mechanisms of mutagenesis are indeed remarkably conserved.

Based upon our *in vivo* studies with protease-deficient strains of *E. coli,* we hypothesized that the UmuD and UmuC proteins are substrates for the Lon protease, and that UmuD′ is a substrate for ClpXP. We plan to extend these observations to look at the proteolytic reactions with highly purified Umu and Lon/ClpXP proteins *in vitro*. Such studies may provide insights into the more general mechanisms of protein degradation.

Now that we have soluble UmuD′C in hand, we plan to extensively characterize the biochemical properties of the complex, especially focusing on the interactions of UmuD′C with subunits of DNA polymerase III that appear necessary to facilitate efficient translesion DNA synthesis.

While the studies outlined above will primarily focus on *Escherichia coli* and *Xenopus laevis*, we plan to characterize a putative UmuC-like homolog that has recently been identified in *Saccharomyces cerevisiae.* Given that repair mechanisms seem to be evolutionarily conserved (especially between yeast and humans), such studies may provide clues as to the role of these homologs in human cells.

B. *DNA REPAIR*

We will pursue the notion that the DDB complex may have a role in addition to recognition of UV lesions. One candidate role is transcription coupling, given our increasing awareness that stalled transcription induces expression of DNA repair genes, and that other XP proteins may serve as subunits of transcription factors. We shall also pursue the notion that the DDB complex may have a particularly high affinity for 6-4 photoproducts as opposed to other UV lesions, i.e., cyclobutane pyrimidine dimers. It may be that the XPA protein functions as a general damage-recognition protein, analogous to the *E.coli* Uvr A protein, but that other proteins serve as accessory factors in the recognition of specific DNA conformations. Therefore, we plan to compare the affinity of DDB versus XPA with respect to binding to a probe containing only single 6-4 lesions. Our work will lean heavily on the use of purified DDB complex proteins (p127, p48, RPA) in the *in vitro* NER reconstituted system.

PUBLICATIONS

Avantaggiati ML, Carbone M, Graessman A, Howard B, Nakatani Y, Levine AS. The SV40 large T antigen and adenovirus E1a oncoproteins interact with distinct isoforms of the transcriptional coactivator, p300. Embo J 1996;15:2236-2248.

Bruck I, Woodgate R, McEntee K, Goodman MF. Purification of a soluble UmuD'C complex from *Escherichia coli*: cooperative binding of UmuD'C to single-stranded DNA. J Biol Chem 1996;271:10767-10774.

Carbone M, Rizzo P, Procopio A, Giuliano M, Pass HI, Gebhardt MC, Mangham C, Hansen M, Malkin DF, Bushart G, Pompetti F, Picci P, Levine AS, Bergsagel JD, Garcea RL. SV40-like sequences in human bone tumors. Oncogene 1996;13:527-535.

Efrati E, Tocco G, Bruck I, Eritja R, Woodgate R, Wilson SH, Tower J, Goodman MF. Biochemical analysis of possible deviations from the "A rule": specificity of nucleotide insertion at abasic DNA template lesions by pol β. In: Hagen U, Jung H, Streffer C eds. Radiation Research, vol 2. New York:Taylor and Francis, 1996; in press.

Frank EG, Ennis DG, Gonzalez M, Levine AS, Woodgate R. Regulation of SOS mutagenesis by proteolysis. Proc Natl Acad Sci USA 1996;93:10291-10296.

Frank EG, Gonzalez M, Ennis DG, Levine AS, Woodgate R. *In vivo* stability of the Umu mutagenesis proteins: a major role for RecA. J Bacteriol 1996;178:3550-3556.

Hixon ML, Lewis AM Jr., Levine AS, Chattopadhyay SK. Limited diversity in the MHC class II loci of Syrian hamster DNA. Lab Anim Sci 1996; in press.

Koch WH, Kopsidas G, Meffle B, Levine AS, Woodgate R. Analysis of chimeric UmuC proteins: identification of regions in *Salmonella typhimurium* UmuC important for mutagenic activity. Mol Gen Genet 1996;251:121-129.

Koch WH, Woodgate R. The SOS response. In: Hoekstra MF, Nickoloff JA eds. DNA damage and repair: molecular and cell biology. Totowa, New Jersey:Humana Press, in press.

Kulaeva OI, Koonin EV, McDonald JP, Randall SK, Rabinovich N, Connaughton JF, Levine AS, Woodgate R. Identification of a DinB/UmuC homolog in the archeon *Sulfolobus solfataricus*. Mutat Res 1996; in press.

Lawrence CW, Borden A, Woodgate R. Analysis of the mutagenic properties of the UmuDC, MucAB and RumAB proteins, using a site specific abasic lesion. Mol Gen Genet 1996;251:493-498.

Lawrence CW, Gibbs P, Woodgate R, Borden A, Szekeres E, Nelson J, Hinkle D. Mutagenic mechanisms in yeast and *E. coli*: studies with individual UV photoproducts and abasic sites. In: Hagen U, Jung H, Streffer C eds. Radiation Research, vol 2. New York:Taylor and Francis, 1996; in press.

Oda N, Levin JD, Spoonde AY, Frank EG, Levine AS, Woodgate R, Ackerman EJ. Arrested DNA replication in *Xenopus* and release by *E. coli* mutagenesis proteins. Science 1996;272:1644-1646.

Peat TS, Frank EG, McDonald JP, Levine AS, Woodgate R, Hendrickson WA. Structure of the UmuD' protein and its regulation in response to DNA damage. Nature 1996;380:727-730.

Peat TS, Frank EG, Woodgate R, Hendrickson WA. Production and crystallization of a selenomethionyl variant of UmuD', an *Escherichia coli* SOS response protein. Proteins 1996;25:506-509.

Peat TS, Frank EG, McDonald JP, Levine AS, Woodgate R, Hendrickson WA. The UmuD′ protein filament and its potential role in damage induced mutagenesis. Structure 1996; in press.

Pompetti F, Rizzo P, Simon RM, Friedlin B, Mew DJY, Pass HI, Picci P, Levine AS, Carbone M. Oncogene alterations in primary, recurrent and metastatic human bone tumors. J Cell Biochem 1996; in press.

Szekeres ES Jr, Woodgate R, Lawrence CW. Substitution of *mucAB* or *rumAB* for *umuDC* alters the relative frequencies of the two classes of mutations induced by a site-specific T-T cyclobutane dimer and the efficiency of translesion DNA synthesis. J Bacteriol 1996;178:2559-2563.

Watanabe-Akanuma M, Woodgate R, Ohta T. Enhanced Generation of A:T->T:A Transversions in a *recA730 lexA51*(Def) Mutant of *Escherichia coli.* Mutat Res 1996; in press.

Woodgate R, Levine AS. Damage inducible mutagenesis: recent insights into the activities of the Umu family of mutagenesis proteins. In:Lindahl T ed. Cancer Surveys: genetic instability in cancer. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1996; in press.

PB

NICHD Annual Report
October 1, 1995 to September 30, 1996
Perinatology Research Branch

The Perinatology Research Branch conducts clinical and laboratory research in maternal and fetal diseases responsible for perinatal morbidity and mortality. The Branch emphasizes a multi-disciplinary approach using expertise from clinical sciences (maternal fetal medicine, neonatology and perinatal pathology), as well as basic sciences and epidemiology, to improve the etiologic understanding, diagnosis, treatment and prevention of disorders related to infant mortality. Under the direction of Roberto Romero, congenital anomalies and causes of prematurity are studied. These two conditions are the leading causes of perinatal morbidity worldwide and are major contributors to infant mortality.

ENDOSCOPIC INTRAUTERINE SURGERY IN THE HUMAN FETUS

The Perinatology Research Branch continues its pioneering work in the development and applications of fetal endoscopic surgery in humans. Over the past four years, the Branch pioneered the utilization of thin-gauge endoscopy in the diagnosis of fetal congenital anomalies and endoscopic fetal surgery for the treatment of disorders of multiple gestation (Twin Reversal Arterial Perfusion Sequence; TRAP) and fetal obstructive uropathy. In collaboration with Dr. Ruben Quintero of Wayne State University, the Branch has developed techniques to conduct in utero fetal endoscopic surgery of the urinary tract and treat vascular disorder of multiple gestations. This work was conducted at the Fetal Diagnostic and Treatment Center at the Detroit Medical Center, Detroit, Michigan.

This year the Branch reported its experience in the utilization of percutaneous umbilical cord ligation in the management of monochorionic multiple gestations with complications such as the "twin to twin transfusion syndrome" and TRAP sequence. Fifteen patients were referred to evaluation and possible treatment. Two were excluded because of misdiagnosis. Of the remaining patients there were 10 with TRAP sequence, one discordant twin with acrania and 2 cases with twin to twin syndrome with a hydropic twin. All cases were evaluated at Hutzel Hospital/Wayne State University where they were referred for treatment. Umbilical cord ligation was performed with a combined endoscopic and sonographic approach in 11 of 13 cases (84% of cases). An average of 8 weeks was gained after the procedure. Seven of the 11 patients undergoing cord ligation had a living child.

Endoscopic devascularization of a large chorioangioma of the placenta was also accomplished endoscopically for the first time. Chorioangiomas are hamartomas which occur in 1% of placentas. Large chorioangiomas have been associated with severe complications often related to a high output fetal cardiac failure. Complications may include polyhydramnios, non-immune hydrops, cardiomegaly, growth retardation, preterm labor and fetal death (16%). Maternal complications include toxemia, abruption placenta and fetal-maternal transfusion. A patient with a large chorioangioma and severe fetal cardiac failure was referred to Hutzel Hospital/Wayne State University at 24 weeks gestation. The fetus was critically ill with signs of impeding fetal death (i.e., reversal of flow in the ductus venosus). Endoscopic ligation of the vessel was accomplished successfully but the fetus died on the third post-operative day. This case illustrates the feasibility of performing this surgical procedure with minimally invasive surgery and opens the possibility that early identification and treatment before fetal cardiac failure may be life-saving.

INFECTION, PREMATURITY, PERIVENTRICULAR LEUKOMALACIA AND CEREBRAL PALSY

The Branch continues its work in the pathophysiology of preterm labor. The focus has been the relationship between subclinical intrauterine infection and preterm labor. A major development in the last two years has been the realization that subclinical intrauterine infection is a risk factor for the development of periventricular leukomalacia (PVL) and cerebral palsy.

PVL is a cerebral lesion characterized by foci of necrosis in the white matter near the lateral ventricles found in 7-26% of neonates with a birthweight under 1,500 g. Between 47% and 80% of these infants developed abnormalities of neuromuscular tone and posture [i.e., cerebral palsy (CP)] , and often cognitive impairment. Inflammatory cytokines released during the course of intrauterine infections have been implicated in the genesis of brain white matter lesions and subsequent CP. The Branch completed a study to determine if fetuses who subsequently were diagnosed to have periventricular brain white matter lesions within the first seven days of life could be identified by determining the concentrations of inflammatory cytokines in amniotic fluid. Women with complicated preterm gestations underwent amniocentesis for clinical indications. Amniotic fluid concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and the natural interleukin-1 receptor antagonist (IL-1ra) were determined by immunoassay. Periventricular white matter lesions of the neonate were diagnosed by neurosonography. Ninety-four women and their neonates were included in the study; white matter lesions were diagnosed in 24% (23/94) of the newborns. The mothers of newborns with brain white matter lesions had higher median concentrations of TNF-α, IL-1β, and IL-6 (but not IL-1ra) in amniotic fluid than those who delivered newborns without white matter lesions. Acute histologic chorioamnionitis was more common in the placentas of neonates with white matter lesions than in those without these lesions. Neonates with white matter lesions were delivered at a lower mean gestational age and birth weight and had a higher rate of significant complications (including respiratory distress syndrome, intraventricular hemorrhage, infection-related complications) than those without white matter lesions. The differences in median IL-1β and IL-6 between these two groups remained significant after adjusting for gestational age and birth weight (IL-6: odds ratio, 5.7; 95% confidence interval, 1.3 to 24.4; IL-1β: odds ratio, 4.4; 95% confidence interval, 1.1 to 17.0). Of the 94 newborns included in this study, 11 died before six months of age and seven developed severe spastic paresis of at least one lower extremity; all seven had white matter lesions and elevated cytokines in amniotic fluid. This data provides strong support for the hypothesis that sub-clinical intrauterine infection leads to the release of inflammatory cytokines. These observations are significant because sub-clinical intrauterine infection may represent a treatable cause of cerebral palsy.

PERINATOLOGY RESEARCH BRANCH

Chief, Roberto Romero, M.D.

Senior Fellows
Neil Athayde, M.D.
Ricardo Gomez, M.D.
Catherine Spong, M.D.
Jorge Tolosa, M.D.

Post-Doctoral Fellows
Jose Cohen, M.D.
Cristina David, M.D.
Fabio Ghezzi, M.D.

Senior Technical Specialist
Samuel Edwin

Romero R. Prenatal medicine: the child is the father of the man. Prenatal and Neonatal Medicine: 1 (1996) 8-11.

Quintero RA, **Romero R**, Reich H, Goncalves L, Johnson MP, Carreno C, Evans MI. *In utero* percutaneous umbilical cord ligation in the management of complicated monochorionic multiple gestations. Ultrasound Obstet Gynecol. 8 (1996) 16-22.

Quintero RA, Reich H, **Romero R**, Johnson MP, Goncalves L, Evans MI. *In utero* endoscopic devascularization of a large chorioangioma. Ultrasound Obstet Gynecol. 8 (1996) 48-52.

Yoon BH, Jun KJ, Park KH, Choi JH, Kim IO, **Romero R**. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1β and tumor necrosis factor-α) and neonatal brain white matter lesions. American Journal of Obstetrics and Gynecology (submitted).

Project Numbers - Annual Report 1996

PERINATOLOGY BRANCH (PB)

Z01 HD 02400-05	The Role of Subclinical Infection and Cytokines in Preterm Parturition Roberto Romero, M.D.
Z01 HD 02401-04	Prenatal Diagnosis of Congenital Anomalies Roberto Romero, M.D.

Z01 HD 2402-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HD 2400-05PB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The role of subclinical infection and cytokines in preterm parturition

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Roberto Romero, M.D.	Head	PB, NICHD
Others:		
Ricardo Gomez, M.D.	Visiting Fellow	PRB, NICHD
Jorge Tolosa, M.D.	Visiting Fellow	PRB, NICHD

COOPERATING UNITS *(if any)*

Department of Obstetrics and Gynecology, National University of Seoul, South Korea (Dr. Bo Yoon).

LAB/BRANCH

Perinatology Research Branch

SECTION

INSTITUTE AND LOCATION

NICHD, Bethesda, 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2		

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
 ☒ (a1) Minors
 ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to explore the relationship between sub-clinical intramniotic infection, preterm parturition and the complications of prematurity. This year the Branch focused specifically on the relationship between sub-clinical intrauterine infection and periventricular leukomalacia.

Periventricular leukomalacia (PVL), a cerebral lesion characterized by foci of necrosis in the white matter near the lateral ventricles is found in 7-26% of neonates with a birthweight under 1,500 g. Between 47% and 80% of these infants developed abnormalities of neuromuscular tone and posture [i.e., cerebral palsy (CP)] , and often cognitive impairment. Inflammatory cytokines released during the course of intrauterine infections have been implicated in the genesis of brain white matter lesions and subsequent CP. The Branch completed a study to determine if fetuses who subsequently were diagnosed to have periventricular brain white matter lesions within the first seven days of life could be identified by determining the concentrations of inflammatory cytokines in amniotic fluid. Women with complicated preterm gestations underwent amniocentesis for clinical indications. Amniotic fluid concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and the natural interleukin-1 receptor antagonist (IL-1ra) were determined by immunoassay. Periventricular white matter lesions of the neonate were diagnosed by neurosonography. Ninety-four women and their neonates were included in the study; white matter lesions were diagnosed in 24% (23/94) of the newborns. The mothers of newborns with brain white matter lesions had higher median concentrations of TNF-α, IL-1β, and IL-6 (but not IL-1ra) in amniotic fluid than those who delivered newborns without white matter lesions ($P<0.01$ for each). Acute histologic chorioamnionitis was more common in the placentas of neonates with white matter lesions than in those without these lesions ($P=0.001$). Neonates with white matter lesions were delivered at a lower mean gestational age and birth weight and had a higher rate of significant complications (including respiratory distress syndrome, intraventricular hemorrhage, infection-related complications) than those without white matter lesions. The differences in median IL-1β and IL-6 between these two groups remained significant after adjusting for gestational age and birth weight (IL-6: odds ratio, 5.7; 95% confidence interval, 1.3 to 24.4; IL-1β: odds ratio, 4.4; 95% confidence interval, 1.1 to 17.0). Of the 94 newborns included in this study, 11 died before six months of age and seven developed severe spastic paresis of at least one lower extremity; all seven had white matter lesions and elevated cytokines in amniotic fluid. These data provides strong support for the hypothesis that inflammatory cytokines released during the course of intrauterine infection play a role in the genesis of brain white matter lesions.

PROJECT TITLE: The role of subclinical infection and cytokines in preterm parturition

PROJECT DESCRIPTION:

The long term goal of this project is the prevention of preterm birth, the leading cause of perinatal morbidity and mortality worldwide. The short term objective is to increase understanding of the basic mechanisms of disease responsible for preterm parturition.

The specific objective for this year was to examine the relationship between umbilical cord cytokine concentrations and the subsequent development of periventricular leukomalacia.

Methods employed:

1. Transabdominal amniocentesis to retrieve amniotic fluid.
2. Sensitive and specific immunoassays for macrophage derived cytokines.
3. Neonatal neurosonography

Progress:

Intrauterine Infection, Cytokines and Cerebral Palsy: Periventricular leukomalacia (PVL), a cerebral lesion characterized by foci of necrosis in the white matter near the lateral ventricles, is found in 7 to 26% of babies with birth-weight under 1,500g. Forty-seven to 80% of these infants developed abnormalities of neuromuscular tone and posture and often cognitive impairment. Although the etiology of this lesion has not been defined, clinical risk factors include prematurity, asphyxia, respiratory distress requiring mechanical ventilation and septicemia. Recently, the role of infection in the pathogenesis of PVL has become a major focus of attention because neonates with sepsis or those born to mothers with documented infection are at increased risk for PVL. A role for cytokines and specifically tumor necrosis factor (TNFα) has been recently postulated in the pathophysiology of PVL. To explore this hypothesis, the Branch conducted a study to examine the relationship between umbilical cord plasma TNFα, interleukin-6 (IL-6) and IL-1 receptor antagonist (IL-1ra) and the occurrence of PVL within the first 3 days of life in 172 preterm births. Plasma concentrations of IL-6, but not of TNF, interleukin-1β (IL-1β) and IL-1ra, were significantly higher in neonates with PVL-associated lesions than in those without these lesions. Moreover, we found that after correcting for other confounding variables IL-6 was an independent predictor of neonatal PVL-associated lesions. Our findings support the hypothesis that intrauterine fetal infection may lead to activation of the cytokine network, white matter damage and PVL. The lack of a relationship between umbilical cord plasma TNFα and subsequent development of PVL does not exclude a role for this cytokine in the development of PVL. This finding may be due to the differential kinetic pattern of plasma cytokine concentrations in response to infection. In animals as well as in human volunteers the administration of lipopolysaccharide (LPS) leads to an increase in plasma concentrations of TNFα, followed by IL-1β and then IL-6. However, the increase in plasma IL-6 concentrations occurs at a time when TNFα concentrations have returned to nearly normal or non-detectable levels. Therefore, it is possible that an intrauterine infection stimulated fetal TNF production, which in turn led to brain white matter damage, although evidence of TNF excess may not be detectable at the time of birth. An elevated IL-6 level in the umbilical cord plasma may be a marker of previous stimulation of the feto-placental immune system by microbial products. An important clinical implication of our study is that IL-6 determinations in umbilical cord plasma represent a simple means of identifying neonates at risk for PVL and subsequent cerebral palsy.

This year the Branch completed a study to determine if fetuses who subsequently were diagnosed to have periventricular brain white matter lesions within the first seven days of life could be identified by determining the concentrations of inflammatory cytokines in amniotic fluid. Women with complicated preterm gestations underwent amniocentesis for clinical indications. Amniotic fluid concentrations of TNFα, IL-1β, IL-6, and IL-1ra were determined by immunoassay. Periventricular white matter lesions of the neonate were diagnosed by neurosonography. Ninety-four women and their neonates were included in the study; white matter lesions were diagnosed in 24% (23/94) of the newborns. The mothers of newborns with brain white matter lesions had higher median concentrations of TNFα, IL-1β, and IL-6 (but not IL-1ra) in amniotic fluid than those who delivered newborns without white matter lesions ($P<0.01$ for each). Acute histologic chorioamnionitis was more common in the placentas of neonates with white matter lesions than in those without these lesions ($P=0.001$). Neonates with white matter lesions were delivered at a lower mean gestational age and birth weight and had a higher rate of significant complications (including respiratory distress syndrome, intraventricular hemorrhage, infection-related complications) than those without white matter lesions. The differences in median IL-1β and IL-6 between these two groups remained significant after adjusting for gestational age and birth weight (IL-6: odds ratio, 5.7; 95% confidence interval, 1.3 to 24.4; IL-1β: odds ratio, 4.4; 95% confidence interval, 1.1 to 17.0). Of the 94 newborns included in this study, 11 died before six months of age and seven developed severe spastic paresis of at least one lower extremity; all seven had white matter lesions and elevated cytokines in amniotic fluid. These data provide strong support for the hypothesis that inflammatory cytokines released during the course of intrauterine infection play a role in the genesis of brain white matter lesions.

Significance:

Prematurity is the leading cause of perinatal morbidity and mortality worldwide. At least 75% of perinatal deaths not due to congenital anomalies are associated with prematurity. Moreover, survivors are at an increased risk for long term neurologic and developmental handicaps. Infants born at < 900g cost society more than they are likely to earn in their life time. Thus, prematurity is considered the single most important problem in perinatal medicine today. Despite the availability of tocolytic therapy (pharmacologic agents to stop uterine contractions) and government funded programs to improve maternal nutrition and prenatal care, the preterm delivery rate in this country has remained essentially unchanged at approximately 9%. This represents at least 180,000 preterm births per year.

Studies performed by our group indicate that at least 25% of all preterm births are associated with subclinical microbial invasion of the amniotic cavity. Moreover, women with subclinical intrauterine infections are refractory to tocolysis and their neonates are at higher risk for perinatal complications. Thus, this group of patients contributes significantly to perinatal morbidity and mortality associated with preterm birth. The studies conducted by this laboratory are designed to define the role of microbial invasion in preterm birth, to elucidate the cellular and molecular mechanisms responsible for preterm birth, and to develop diagnostic and therapeutic modalities for the treatment of preterm labor and prevention of preterm birth.

Prematurity is the leading identifiable cause of cerebral palsy. Periventricular leukomalacia (PVL), a cerebral lesion characterized by foci of necrosis in the white matter near the lateral ventricles, is found in 7 to 26% of babies with birth-weight under 1000g and is a major risk factor for cerebral palsy. The Branch has demonstrated that there is a relationship between subclinical infection and the subsequent development of PVL and that neonates at risk for PVL can be identified by an elevated concentration of IL-6, a cytokine in umbilical cord blood and amniotic fluid.

By far the most important implication of this work is the identification of intrauterine infection, a condition treatable with antibiotics, as a cause of a chronic, life-long disabling disease: cerebral palsy. This realization opens doors for early diagnosis, treatment and prevention of neurologic disability.

REFERENCES:

Yoon BH, Jun KJ, Park KH, Choi JH, Kim IO, **Romero R**. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1β and tumor necrosis factor-α) and neonatal brain white matter lesions, (submitted).

Mazor M, Chaim W, Horowitz S, ROMERO R, Glezerman M. The biomolecular mechanisms of preterm labor in women with intrauterine infection. Isr J Med Sci 1994; 30:317-22

Mazor M, Chaim W, ROMERO R. Preterm labor syndrome. Harefuah 1995; 128:111-16

Horowitz S, Mazor M, ROMERO R, Horiwitz J, Glezerman M. Infection of the amniotic cavity with Ureaplasma urealyticum in the midtrimester of pregnancy. J Reprod Med 1995; 40:375-79.

Romero R, Munoz H, Gomez R, Galasso M, Sherer D, Cotton D, Mitchell M. Does infection cause premature labor and delivery? Semin Reprod Endocrinol 1994; 12:227-48

Romero R, Munoz H, Gomez R, Parra M, Polanco M, Valverde J, Hasbun J, Garrido J, Cotton DB, Mitchell MD. Prostaglandins: Cause or consequence of labor? Abstract #590, presented at the 15th Annual Meeting of the Society of Perinatal Obstetricians, January, 1995

Romero R, Munoz H, Gomez R, Sherer DM, Ghezzi F, Ghidini A, Alfi O, DeVore G, Randolph L. Two-thirds of spontaneous abortion/fetal deaths after genetic midtrimester amniocentesis are the result of a pre-existing subclinical inflammatory process of the amniotic cavity. Abstract #24, presented at the 15th Annual Meeting of the Society of Perinatal Obstetricians, January, 1995

Yoon B, **Romero R**, Yang SH, Jun JK, Choi JH, Kim IO, Syn HC, Kim SW. Interleukin-6 concentrations in umbilical cord plasma identify infants at risk for the development of white matter brain lesions associated with periventricular leukomalacia. Abstract #37, presented at the 15th Annual Meeting of the Society of Perinatal Obstetricians, January, 1995

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HD 2401-04 PB

PERIOD COVERED

October 1, 1995 to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prenatal Diagnosis of Congenital Anomalies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Roberto Romero, M.D.	Head	PRB, NICHD
Others:		
Ricardo Gomez, M.D.	Visiting Fellow	PRB, NICHD
Jorge Tolosa, M.D.	Senior Staff Fellow	PRB, NICHD

COOPERATING UNITS *(if any)*

Department of Obstetrics and Gynecology, Wayne State University (Ruben Quintero, MD)

LAB/BRANCH

Perinatology Research Branch

SECTION

INSTITUTE AND LOCATION

NICHD, Bethesda 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2		

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The focus of this project is to improve the diagnosis and treatment of fetal disease. Focus is placed on the prenatal diagnosis of congenital anomalies with non-invasive methods (e.g., high resolution ultrasound and color Doppler flow mapping) and invasive methods.

The Branch pioneered the utilization of thin-gauge endoscopy in the diagnosis of fetal congenital anomalies and endoscopic fetal surgery for the treatment of disorders of multiple gestation (Twin Reversal Arterial Perfusion Sequence; TRAP) and fetal obstructive uropathy.

This year the Branch reported its experience in the utilization of percutaneous umbilical cord ligation in the management of monochorionic multiple gestations with complications such as the "twin to twin transfusion syndrome" and TRAP sequence. Fifteen patients were referred to evaluation and possible treatment. Two were excluded because of misdiagnosis. Of the remaining patients there were 10 with TRAP sequence, one discordant twin with acrania and 2 cases with twin to twin syndrome with a hydropic twin. All cases were evaluated at Hutzel Hospital /Wayne State University where they were referred for treatment. Umbilical cord ligation was performed with a combined endoscopic and sonographic approach in 11 of 13 cases (84% of cases). An average of 8 weeks was gained after the procedure. Seven of the 11 patients undergoing cord ligation had a living child.

Endoscopic devascularization of a large chorioangioma of the placenta was also accomplished endoscopically for the first time. Chorioangiomas are hamartomas which occur in 1% of placentas. Large chorioangiomas have been associated with severe complications often related to a high output fetal cardiac failure . These complications include polyhydramnios, non-immune hydrops, cardiomegaly, growth retardation, preterm labor and fetal death (16%). Maternal complications include toxemia, abruption placenta and fetal-maternal transfusion. A patient with a large chorioangioma and severe cardiac failure was referred to Hutzel Hospital/Wayne State University at 24 weeks gestation. The fetus was critically ill with signs of impeding fetal death (i.e., reversal of flow in the ductus venosus). Endoscopic ligation of the vessel was accomplished successfully but the fetus died in the third post-operative day. This case illustrates the feasibility of performing this surgical procedure with minimally invasive surgery and opens the possibility that early identification and treatment before fetal cardiac failure may be life-saving.

PROJECT TITLE: Prenatal Diagnosis of Congenital Anomalies

PROJECT DESCRIPTION:

The Perinatology Research Branch continues its pioneering work in the development of fetal endoscopic surgery in humans. Over the past three years, the Branch, in collaboration with Dr. Quintero from Wayne State University/Hutzel Hospital developed techniques to conduct in utero fetal endoscopic surgery for the treatment of fetal congenital anomalies in twin gestations (e.g., Twin Reversal Arterial Perfusion sequence) and obstructive uropathy.

SPECIFIC AIMS:

1. To perform endoscopic intrauterine fetal surgery in congenital anomalies amenable to correction.

2. To further the knowledge about the natural history, diagnosis, and treatment of congenital anomalies.

METHODS EMPLOYED:

1. High resolution ultrasound and color Doppler flow mapping are two non-invasive techniques already utilized to establish prenatal diagnosis of conditions difficult or impossible to recognize in the past.

2. Transabdominal thin-gauge embryofetoscopy is a technique used to gain access to the fetus and the intrauterine environment. This procedure allows visualization of the surface anatomy of the human embryo and fetus, as well as insertion of surgical instruments into the amniotic cavity under direct visualization. Fetal surgery can thus be performed for the correction of some congenital anomalies.

PROGRESS REPORT:

The Perinatology Research Branch continues its pioneering work in the development of fetal endoscopic surgery in humans. Over the past three years, the Branch, in collaboration with Dr. Quintero from Wayne State University, developed techniques to conduct in utero fetal cystoscopy and endoscopic surgery of the fetal urinary tract and treatment of disorders of monochorionic multiple gestation (i.e., TRAP sequence, twin to twin transfusions, discrepant twins for anomalies).

1.Endoscopic surgery for the treatment of disorders of monochorionic multiple gestation: This year the Branch reported its experience in the utilization of percutaneous umbilical cord ligation in the management of monochorionic multiple gestations with complications such as the "twin to twin transfusion syndrome" and TRAP sequence. Fifteen patients were referred to evaluation and possible treatment. Two were excluded because of misdiagnosis recognized only after referral to us. Of the twelve remaining patients there were 10 with TRAP sequence, one discordant twin with acrania and 2 cases with twin to twin syndrome with a hydropic fetus. All cases were evaluated at Hutzel Hospital/Wayne State University. Umbilical cord ligation was performed with a combined endoscopic and sonographic approach in 11 of 13 cases (84% of cases). An average of 8 weeks was gained after the procedure. Seven of the 11 patients undergoing cord ligation had a living child.

2.Endoscopic devascularization of a chorioangioma of the placenta: Chorioangiomas are hamartomas which occurred in 1% of placentas. Large chorioangiomas have been associated with severe complications often related to a high output fetal cardiac failure . These complications include polyhydramnios, non-immune hydrops, cardiomegaly, growth retardation, preterm labor and fetal death (16%). Maternal complications include toxemia, abruption placenta and fetal-maternal transfusion. A patient with a large chorioangioma and severe cardiac failure was referred to Hutzel Hospital/Wayne State University at 24 weeks gestation. The diagnosis was made with ultrasound and color Doppler demonstration of the vessels. The fetus was critically ill with signs of impeding fetal death (i.e., reversal of flow in the ductus venosus). Endoscopic ligation of the vessels supplying the tumor was accomplished successfully but the fetus died in the third post-operative day. This case illustrates the feasibility of performing this surgical procedure with minimally invasive surgery (endoscopic surgery). This approach may be life-saving in fetuses who are not terminally ill.

SIGNIFICANCE:

Congenital anomalies are the second leading cause of perinatal mortality and are a major focus of research activity for our team. The Perinatology Research Branch has developed a novel method to access the utero-fetal environment: embryofetoscopy. This technique has allowed diagnosis of conditions beyond the resolution of ultrasound (e.g., polydactyly in a first trimester fetus). More importantly, we have proved its feasibility in the in utero treatment of some congenital anomalies amenable to surgical repair. We foresee that future applications will also include access to the embryonic and fetal circulation for therapeutic purposes (e.g., gene therapy). This procedure, though more invasive than amniocentesis and percutaneous fetal blood sampling, has the advantage of avoiding the risks of hysterotomy to correct diseases of the human fetus amenable to surgical treatment.

Ultrasound studies of the fetus and Doppler interrogation of the fetal hemodynamic circulation, including color Doppler flow mapping, have been of value in expanding the number of congenital anomalies which can be prenatally diagnosed, as well as improving the accuracy of gray scale ultrasound.

PROPOSED COURSE:

We plan to conduct the following studies:

1. Expand the diagnostic and therapeutic indications for embryofetoscopy in human pregnancy.

2. Determine the value of ultrasound examination, fetal echocardiography, and Doppler flow studies of the maternal and fetal peripheral vascular system in the prediction of preterm delivery in patients at risk for this complication.

3. Determine the value of sonographically-derived fetal biometry in the second trimester to predict the growth potential of the fetus and evaluate the clinical significance of deviation from the model.

REFERENCES:

Quintero RA, **Romero R**, Reich H, Goncalves L, Johnson MP, Carreno C, Evans MI. *In utero* percutaneous umbilical cord ligation in the management of complicated monochorionic multiple gestations. Ultrasound Obstet Gynecol. 8 (1996) 16-22.

Quintero RA, Reich H, **Romero R**, Johnson MP, Goncalves L, Evans MI. *In utero* endoscopic devascularization of a large chorioangioma. Ultrasound Obstet Gynecol. 8 (1996) 48-52.

Goncalves LF, Sherer DM, Silva M, Ghezzi F, Treadwell MC, ROMERO R. Prenatal sonographic demonstration of agenesis of the right and left portal veins and associated intrahepatic postosystemic shunts. J Ultrasound Med (in press).

Sherer DM, ROMERO R, Silva M, DeVore G, Goncalves LF, Ghezzi F, Cohen J, DeVore GR. Prenatal diagnosis of coarctation of the aorta with Color Doppler Imaging. J Ultrasound Med (in press).

Treadwell MC, Sherer DM, Sacks AJ, Ghezzi F, ROMERO R. Resolution of nonimmune hydrops secondary to fetal hypothyroidism following cordocentesis and adjusted medical therapy. Obstet Gynecol (in press).

Lauria MR, Gonik B, ROMERO R. Pulmonary hypoplasia: Pathogenesis, diagnosis, and antenatal prediction. Obstet Gynecol (in press).

DESPR SUMMARY

NICHD Annual Report

October 1, 1995 to September 30, 1996

Table of Contents
Division of Epidemiology, Statistics and Prevention Research

Office of the Director

A study of maternal and infant deaths, pregnancy outcome and child survival in Pakistan..
Intrauterine growth retardation..
Maternal mortality..
Infant and child development..
A randomized controlled trial for the evaluation of a new prenatal care model..
NICHD-DC infant mortality initiative..

Biometry and Mathematical Statistics Branch

Role of meta analysis in monitoring clinical trials................
Multiple hypothesis testing..
Testing for homogeneity in mixed models............................
Logistic regression of t-year survival probability................
Categorical data analysis..
Nonparametric tests for cross-over trials with repeated measures...
Comparing several treatments to a control with expected variance inflation...
Growth curve analysis..
Methodological research in meta-analysis...........................
Repeated measure method for multivariate outcome data with missing observations...
Missing covariates in logistic regression..........................
A new decision rule in active control equivalence studies..........
Multivariate permutation tests.....................................
Sequential testing with interval censored data.....................
Meta-analysis of trials of calcium for preeclampsia prevention.....
Vasectomy and prostate cancer risk.................................
Clinical trial of calcium for preeclampsia prevention.............
Serum caffeine metabolites and pregnancy outcome...................
Unintended consequences of public cholesterol screening: false reassurance...
Epidemiology of injuries in infants................................
Classification systems for birth weight............................
Infectious disease death in postneonatal infants...................
Time to treatment for hypothyroidism in cystinosis.................
Human growth hormone trials..
Statistical methods for side-to-side difference in nerve conduction studies...................................
Preventing problem behavior among middle school students...........
Intrauterine growth retardation, preterm birth and long term neurodevelopmental outcome...
Maternally linked longitudinal studies, Missouri and Utah..........

Maternal risk factors for intrauterine growth retardation and preterm labor and delivery....................................
Better babies project in the District of Columbia.................
Sperm production in testicles obtained in autopsy.................
Contraceptive development...
ETEC vaccine study in Egypt.......................................
Cholera vaccine trial in Vietnam..................................
Diarrheal morbidity among infants of HIV infected mothers.........
Data analysis support for DIEP study data.........................
Neural tube defect studies in Ireland.............................
Tubal ligation study data analysis................................

Epidemiology Branch

Calcium supplementation trial....................................
Diabetes in pregnancy...
Prostaglandin metabolism in preeclampsia..........................
Maternal caffeine intake..
Reproductive compensation in adolescence..........................
Contextual poverty, individual socioeconomic status and poor pregnancy outcomes in the District of Columbia....................
Impact of changing paternity on pregnancy outcomes................
Very preterm twin birth: role of race and fetal sex...............
Adolescent age at first pregnancy and subsequent obesity..........
Evaluation of the effectiveness of a community-based, enriched model prenatal intervention project in the District of Columbia....
Intergenerational studies...
Effect of maternal diet and nutritional status upon birth weight in a less-developed country.....................................
Surrogate measures of low birth weight in a less-developed country.
Birth weight related to growth and health by age six..............
Survival and health of high-risk deliveries.......................
Low birth weight and infectious disease mortality.................
Intrauterine growth retardation, preterm birth and long-term neurodevelopmental outcomes.......................................
Irish neural tube defects study...................................
Fetal alcohol syndrome studies....................................
Exposure to maternal smoking and childhood cancer.................
Long-term outcomes of leukemia survivors..........................
Drowning..
Childhood injuries in the District of Columbia....................
Medically attended nonfatal injuries in U.S. children and adolescents...
Occupational injuries in U.S. children and adolescents............
International comparisons of injuries in children.................
Injury fatalities in infants.......................................
Protective level of maternal antibody against early-onset Group B streptococcal sepsis in neonates................................
Group B streptococcal colonization during pregnancy...............
Effectiveness of intrapartum antibiotic prophylaxis against early-onset neonatal group B streptococcal disease.................
Gestational syphilis..
Bangladesh trial of killed oral cholera vaccines..................
Trial of killed oral cholera vaccine in Vietnam...................

Cholera vaccination in refugee camps....................................
Protective relationships between Vibrio cholerae 01 and Vibrio cholerae 0139..
Helicobacter pylori in children...
Studies in Egypt..
Studies in Bangladesh...
Evaluation of Vi-conjugate typhoid vaccine in Vietnam...............
Typhoid epidemiology in India...
Age-appropriate immunization in the District of Columbia............
Conventional vaccination of HIV-infected infants....................
Pneumococcal vaccination of HIV-infected infants....................
New statistical models for vaccine trials...........................
New criteria for case-control studies of vaccine effectiveness.....
Establishment of methods for vaccine effectiveness trials..........
Ear infections and respiratory conditions...........................

Prevention Research Branch

Prevention of childhood injury among preschool children............
MD/family partnership: education in asthma self-management.........
Parent-child interaction and asthma self-management................
Preventing problem behavior among middle school students...........
Pregnancy prevention among minority adolescents....................
Immunization among D.C. infants.......................................
Survey of parental behaviors to prevent adolescent alcohol use.....
Perceptions of parents regarding the onset of sexual intercourse among youth..

NICHD Annual Report

October 1, 1995 to September 30, 1996

Division of Epidemiology, Statistics and Prevention Research

The Division of Epidemiology, Statistics and Prevention Research conducts investigations in the field of maternal and child health. Epidemiological projects cover major public health concerns in maternal and child health such as perinatal and infant mortality, intrauterine growth retardation and preterm delivery, birth defects and developmental abnormalities, and also child growth and development. Other studies are focusing on the epidemiology of injury and injury prevention, pediatric infectious diseases, and vaccinology and vaccine trials, as well as clinical trials and community based interventions. Other investigations are designed to promote healthful behaviors and the prevention or amelioration of problems during pregnancy, infancy, childhood and adolescence. These studies make use of a variety of research strategies including surveys, randomized trials, as well as demonstration/education projects.

There is an interest in research in statistical theory and methodology and studies are conducted in these fields of relevance to the problems under investigation in the area of maternal-child health. The program provides consultation in research design and statistics, as well as collaborates with other scientists on statistical and mathematical problems related to specific research projects.

The Division consists of the Biometry and Mathematical Statistics Branch, the Computer Sciences Branch, the Epidemiology Branch and the Prevention Research Branch.

Office of the Director

A Study of Maternal and Infant Deaths, Pregnancy Outcome and Child Survival in Pakistan

A continuing collaborative effort with the Department of Community Health Sciences of the Aga Khan University in Karachi, Pakistan has developed for the first time population based data in different provinces of Pakistan on the prevalence of maternal, infant and child deaths and also on causes of death through verbal autopsies. These surveys have now been completed in three sites in Baluchistan, the City of Karachi, the Province of Sindh and three sites in the Northwest Frontier Province. Also a pregnancy outcome study and a follow up of 1000 births from this project to two years of age has been completed.

Intrauterine Growth Retardation

Considerable progress has been made in the analysis of data from the Pregnancy Outcome Study. These deal with risk factors related to intrauterine growth retardation which is a major risk factor for most of the low birth weight births in developing countries. Analysis of the data suggests that risk factors include socioeconomic conditions, including housing construction, paternal and maternal education, paternal employment and the source of water supply. There are marked differences among the different ethnic groups in these communities which consists of Hindus, Muslims and Christians. Biological factors related to the risk of IUGR include young maternal age, primiparity and high parity, history of poor prior pregnancy outcome, a short interconception interval, low maternal height and weight, as well as a low mid-arm circumference and low skinfold thickness reflecting maternal nutritional status. In addition, there was a marked increase in risk due to consanguinity.

In a multi-variate analysis, the remaining risk factors included the source of water supply, maternal education and paternal employment, primiparity and grand multiparity, consanguinity, less than one year interval between birth to conception, low maternal height and weight and also non-vegetarian diet. These findings suggest possible interventions to improve maternal nutrition but also raising the status of women by mandating primary education of all school age girls.

Maternal Mortality

In an analysis of the contextual determinants of maternal mortality in rural districts of Pakistan, we used data about the availability of health services, clinics, physicians and lady health visitors, as well as modes of transportation and distance to such facilities as contextual variables which may impact upon maternal mortality.

Access to adequate obstetrical care is the essential intervention to reduce maternal deaths in developing countries. Not unexpectedly, distance is a major determinant of mortality and pregnant women at high risk of maternal mortality living in a remote village are more likely to die than pregnant women at comparable risk but closer to a medical facility. There is a relationship between the number of people per primary health care facility and maternal mortality, and an increase in population per primary health care facility is associated with an increase in maternal mortality. There was little evidence that government health services currently in place prevent maternal deaths in the areas of Baluchistan and Northwest Frontier Providence included in the analysis.

Infant and Child Development

An analysis of variables related to stunting in children from birth to two years of age was completed. Approximately 1000 children were followed from birth to 24 months and seen at 1 month, 3 months, 6 months, 9 months, 12 months, 18 months and 24 months. Information was obtained at each visit about intercurrent illness, especially episodes of diarrhea and of upper and lower respiratory tract infection, and infant feeding practices, and also measurements were taken of the child's height and weight and head circumference at each one of these intervals. The rate of stunting in this population is high and increases from a level of 27 percent at 6 months to 52 percent at 12 months. Wasting is relatively low with a rate of 2 percent at 3 months, increasing to 6.6 percent at 12 and 24 months. The risk of stunting was increased by low level of income, and poor building material used for the house construction and lack of running water of electricity. Other factors related were crowding and consanguinity. High maternal and paternal education was protective. Lower maternal height and weight increased the risk of stunting. Stunting was lower among primigravidas. There was a strong correlation between IUGR status at birth and risk of stunting.

Breast feeding, either exclusive or partial breast feeding, was protective at 6 and 12 months and reduced the rate of stunting.

An illness index consisting of frequency of diarrhea, upper and lower respiratory infection and skin rash showed an increase risk of stunting related to illness as compared to those without intercurrent disease.

In a multi-variate model, significant effects are seen for income, water supply and maternal education. Of maternal variables, low maternal weight increased the risk and primiparity decreased the risk of stunting. Prior IUGR status at birth stayed in the model and increased the risk of stunting significantly. The occurrence of diarrhea at 6 months increased the risk with no effect seen at subsequent intervals.

A Randomized Controlled Trial for the Evaluation of a New Prenatal Care Model

We are collaborating with the WHO in a randomized clinical trial comparing two models of antenatal care to establish the relative merits of each model. This trial is being conducted in five sites consisting of Rosario, Argentina; Havana, Cuba; Congella, South Africa; Khan Kaen, Thailand; and Jehda, Saudi Arabia. Randomization is by clinics or other units of prenatal care providers and the clinics will be assigned to the current regimen of prenatal care or the new model to be tested. There are 12 units or clinics per site and the patient population included in this trial across the five sites will be between 30-36,000. The individual clinics or units of randomization are chosen to be similar in numbers of patients attending, and characteristics such as race, ethnicity, religion, socioeconomic status, and health, as well as rates of various perinatal outcomes. The new prenatal care system administered over four visits will include only activities which have been demonstrated to be effective in improving pregnancy and maternal, as well as newborn outcomes. They are specific interventions included in each of the four visits related to specific adverse health conditions identified. In addition, health education information for the mother are the central part of every visit, emphasizing the danger signs of complications during pregnancy and actions that must be taken.

It is expected that recruitment of patients for this trial will take approximately 18 months and if this is a realistic estimate, data would be available for analysis approximately 2 to 2 1/2 years from now. Thus the study should be completed within three years from now.

NICHD-DC Infant Mortality Initiative

The NICHD-DC Infant Mortality Initiative is a collaborative project between six institutions in Washington, D.C., the Research Triangle Institute in North Carolina, and the Division of Epidemiology, Statistics and Prevention Research of the NICHD. The objectives of this project are to conduct investigations which are directly or indirectly related to the high infant mortality rate in Washington, D.C. and may lead to a better understanding of its determinants and to the development and testing of interventions. This project began about four years ago. The collaborating institutions in Washington, D.C. include the Children's Hospital Medical Center, the Commission of Public Health of the District of Columbia Government, the D.C. General Hospital, Georgetown University, Howard University and the University of the District of Columbia. The Research Triangle Institute (RTI) in North Carolina is the data management center for this project. The Director of the Division of Epidemiology, Statistics and Prevention Research (DESPR) is the program officer for this project and as such responsible for its management and its overall scientific direction.

The Initiative is supported through a cooperative agreement, an extramural mechanism, and therefore operates under the rules of a cooperative agreement. This means that a steering committee consisting of the principal investigators of the six participating centers in Washington, D.C. and of RTI, as well as the program officer of NICHD, decides collectively about research topics and directions, as well as priorities in research.

There are about 180 scientists and support personnel involved in various projects from these participating institutions in Washington, D.C. They are collaborating with a number of investigators from the DESPR. There is active participation by investigators from the Epidemiology Branch, the Prevention Research and the Biometry and Mathematical Statistics Branch. At present six protocols are in place and fully operational in Washington, D.C. These studies include an intervention to improve parenting skills among high risk mothers leading to better utilization of preventive health services for their infants; an adolescent pregnancy prevention program which is school based; a study of barriers, motivators and facilitators to the entry and maintenance of prenatal care; a baseline study of injuries among infants and young children leading to emergency room visits in Washington, D.C.; a study of the determinants of lack of age appropriate immunization coverage; and a study of characteristics of health services in neonatal intensive care units among eight hospitals in Washington, D.C.

This collaboration involves a major commitment on the part of several members of the staff of the DESPR to insure day-to-day coordination, management and direction, and to provide scientific input into protocols of interest to the scientific staff of the DESPR, and participate in the implementation, completion, analysis and publications of these studies.

Details on specific protocols which involve scientific staff from DESPR collaborating with participating institutions in Washington, D.C. are provided in the summary reports of the Branches.

Biometry and Mathematical Statistics Branch

The Biometry and Mathematical Statistics Branch (BMSB) has been active in methodological research and providing institute-wide statistical consulting. As for the personnel action, Dr. Eunsik Park joined the BMSB in March as a visiting fellow after her Ph.D. in mathematical statistics from Seoul National University, Seoul, Korea, and will stay with the BMSB for two years. There has been no other personnel action this year. Dr. Kai Yu continues to work toward the problem behavior prevention project, NIH-DC initiatives to reduce infant mortality, small-for-gestation age study, and maternally linked longitudinal data analysis in addition to his methodological research. Dr. Yu has been invited to present his work in Norway where he will present a talk on intrauterine growth analysis on ultrasound measurements at the Successive Small-for Gestational Age Studies Meetings in Trondheim, Norway in September. The CPEP trial is over and the final data analysis is in progress under Dr. Rebecca DerSimonian's direction. She made an invited presentation on meta-analysis of trials of calcium for preeclampsia prevention at the Tenth World Congress: International Society for the Study of Hypertension in Pregnancy at Seattle in August. Dr. James Troendle made invited presentations on "A permutational step-up method of testing multiple outcomes" at the International Conference on Multiple Comparisons in Tel Aviv, Israel in June and also at the September 4 Division of Cancer Prevention and Control Colloquium of the National Cancer Institute. Dr. Young Jack Lee made invited presentations on roles of biostatistics in medicine at the University of Missouri at Columbia in April and the Chung-ang Medical Center in Seoul, Korea in August. The BMSB hosted three visitors from Korea during the summer: Dr. Taesung Park, a former BMSB visiting fellow, Dr. Sin-ho Jung, and Dr. Chang-gok Chang. Dr. Lee has been invited to write an article on testing ordered alternatives for Encyclopedia of Biostatistics to be published by the Wiley Publication.

Statistical Research

We will discuss selected statistical research projects completed or ongoing.

Role of Meta Analysis in Monitoring Clinical Trials

Statistics in Medicine published in May the proceedings of the conference on the role of meta analysis in monitoring clinical trials that was organized by Dr. Lee and held in Bethesda in June 1994. The proceedings will provide a valuable guidance for using external evidence in designing and monitoring clinical trials.

Multiple Hypothesis Testing

Dr. Troendle has done extensive research in multiple testing. He has recently developed a permutational step-up method of testing multiple outcomes. This method requires only that the univariate tests be consistent, and is valid regardless of the correlation between outcomes. The method performs with high power relative to competing methods which are either too conservative (Bonferroni methods) or require extreme distribution and correlation assumptions (normal theory). The method is more computational than the step-down method developed by Westfall and Young and Troendle, but when many false null hypotheses are being tested step-up tests generally outperform step-down tests. A paper has been accepted for publication in Biometrics.

In collaboration with Dr. Julie Legler of National Cancer Institute, Dr. Troendle has written a paper comparing the two major testing approaches to multiple outcome data analysis. The first is to apply a global test, and if significant to then proceed via the closed testing algorithm to test all subsets of the outcomes. The second method is to order the individual outcomes by observed significance, and use a stepwise test which allows one to assert that the global hypothesis is false if any of the individual hypotheses are rejected. The paper uses extensive simulation results for multivariate normal data to compare the existing methods with some new methods of one sided testing. The comparison encompasses the family wise error rate control, power, and computation.

Finally, Dr. Troendle has developed both a normal theory and permutation based stepwise testing procedure which control the false discovery rate (FDR). The normal theory method is applicable for the general multiple testing problem when the test statistics have a common correlation coefficient. The permutation methods are for the comparison of two groups for each of several outcomes. The methods can be shown quite generally to asymptotically control the FDR.

Simulations indicate that the methods are preferable to the Bonferroni type test which is currently the only known FDR controlling procedure.

Testing for homogeneity in mixed models

Medical research often involves data which may not represent a sample from a homogeneous population. For example, placebo rates or treatment effects from a multi-center randomized clinical trial may vary across the clinics if their underlying populations differ, e.g. different distributions of age or race. This is especially so for rates and treatment effects in a meta-analysis of related clinical trials evaluating the efficacy of a certain therapy in different risk groups. This research describes several tests to assess the homogeneity model against various alternative models that imply heterogeneity under a mixture model. A simulation study evaluates the performance of these tests in a simulation of samples from mixtures of Gaussian, binomial and Poisson distributions. Drs. Rebecca DerSimonian and Kai Yu collaborate on this methodological research.

Logistic Regression of t-year Survival Probability

Drs. Young Jack Lee and Sinho Jung have been working on generalizing the logistic regression method to the survival data analysis. While the Cox survival regression method introduces bias into parameter estimates for non-proportional hazard survival data, the proposed method can be applied to the broad class of survival data with little or no bias in parameter estimates. The proposed method is less efficient than the Cox method under the proportional hazard model. The proposed method, however, is more broadly applicable because it does not require a specific model assumption. A paper has been tentatively accepted for publication in the Journal of American Statistical Association.

Categorical Data Analysis

Dr. Kai Yu has proposed a simple measure of comparison for two sequences of probabilities. The proposed measure is the squared distance between these two sequences. An estimation procedure including a point estimator and a confidence interval is proposed for this simple measure. A procedure is presented for testing the difference of the two sequences. This new measure and the statistical procedures based on it have certain distinct advantages over the Mantel-Haenszel procedure in the situation where one sequence is not majorized by the other. A test which takes advantages of both the squared distance test and the Mantel-Haenszel test is proposed. Some simulation studies on these three varieties are conducted. Results have been published in Journal of Statistical Planning and Inference.

Dr. Yu has been working on a family of easily computable estimators of the common odds ratio of a number of two by two contingency tables. The well-known mantel-Haenszel estimator as a special case. A necessary and sufficient condition is given for the consistency of the estimators for the case when the tables are sparse and the number of tables becomes large. Variance estimators will also be studied. Results have been published in the Canadian Journal of Statistics.

Ahn and Odom-Maryon (1995) have made a study of the estimation of the common odds ratio of K 2x2 contingency tables under the beta-binomial sampling scheme. They have recommended that if the data within a cluster are positively correlated, the

Mantel-Haenszel estimator should be used with an adjusted variance. Dr. Yu complements Ahn and Odom-Maryson study by considering the case where the numbers of clusters and the cluster sizes may be different. More importantly Dr. Yu has developed the variance of the asymptotic distribution of the Mantel-Haenszel estimator as the number of tables becomes large while the numbers of clusters and the cluster size of each table is bounded. Results are published in *Statistics in Medicine* as a letter to editor.

Nonparametric tests for cross-over trials with repeated measures

In the NICHD osteogenesis imperfecta study, ten participating children were randomized into two groups. In one group of five OI children, the subjects were observed for sixteen months while braced, and observed for another sixteen months while unbraced. In another group, the order of being braced and being unbraced was reversed. Each subject made four return visits per 16 month period of observation. The amount of sleeping time was an outcome variable measuring each child's activity level. If the data are normal and no observations are missing, then the split-plot method is the method of choice. However, some children missed visits and the outcome variable was non-normal. Drs. Haesook Kim (contract statistician) and Young Jack Lee have proposed a nonparametric method based on ranks of observations within each child to test the cross-over effect. If the cross-over effect is not significant, then the treatment effect can be tested without bias. However, if the cross-over effect is significant, then the treatment effect can be tested based only on observations from the first sixteen month observation period. This research will be presented at the 1996 NIH research festival as a poster presentation.

Comparing Several Treatments to a Control With Expected Variance Inflation

Dr. Troendle, working with Cliff Blair of University of Southern Florida, has completed a paper which gives parametric and non-parametric tests for comparing several treatments to a control when it is expected that the treatments will increase the variability. A generalization of the Behrens-Fisher problem is considered where there are k (2) treatments being compared to a control and it is thought that the variance among each treatment group will exceed (or equal) that of the control group. In this case a modified F test has been advocated, but has shortcomings when k exceeds two and the variances of the treatment groups is not inflated. A combination procedure which avoids this problem is proposed, and a likelihood ratio test is also computed. Both new procedures have a preferable power profile for certain data distributions.

Growth curve analysis

Dr. Yu, in collaboration with Dr. Allen Herman, has been studying the growth curve has been motivated by the ultrasound measurements in the intrauterine growth in the Small-for-Gestational Age Study in Alabama and Scandinavia. Dr. Yu is developing various parametric and nonparametric procedures to analyze the data. Review of the mathematical and statistical models is conducted. Dr. Yu will present part of this research in Norway in September.

Methodological Research in meta-analysis

Dr. DerSimonian, a leading researcher in meta-analysis method, has been studying the role of meta-analysis in design and monitoring of clinical trials. This research involves several topics which were motivated by the Trial of Calcium Supplementation for the Prevention of Preeclampsia (CPEP). Dr. DerSimonian is the primary statistician for the CPEP study. The topics were prompted by several meta-analyses which indicated a large effect of calcium for preeclampsia prevention. A member of the Data and Safety Monitoring Board for CPEP strongly argued for analyzing interim data incorporating these large effects from the previous studies. Other members disagreed equally strongly. This research, therefore, addresses the role of external data (i.e., estimates from a meta-analysis) in the design, monitoring, and reporting of results for CPEP.

Dr. DerSimonian also works on to establish standards for proper meta-analyses and their reporting. Meta-analysis of clinical trials have become increasingly common in the medical literature. Yet, in several other examples, large effects concluded by the meta-analysts have been in conflict with data from a subsequent large trial. These include the trials of aspirin for preeclampsia prevention and trials of magnesium for myocardial infarction. Such apparent conflicts often result because the-meta-analysis fail to adequately account for heterogeneity of effects across trials. This study evaluates the practice of meta-analysis of clinical trials published in medical journals to identify common problems that yield confusing or even misleading conclusions about treatment effects. Based on recent empirical evidence from general medical journals, this research will propose standards for proper meta-analyses and their reporting.

Repeated measure method for multivariate outcome data with missing observations

In studying the effect of tubal sterilization on serum estradiol and pregnanediol during the luteal period in reproductive-aged women, blood samples were drawn three times approximately during the luteal period. Blood samples were taken at the baseline, 1 months, 1 year and 2 years return visit. An actual distribution of days of taking blood samples is spread between day -10 and day +20 of the ovulation day. This implies that a study individual has a 30-variate observation vector with 27 variates' having missing values. The primary question was to determine if the serum estradiol level in tubal sterilized women is significantly different from unsterilized women. In modeling the correlation structure of the multivariate repeated measure data, we have to consider two different correlation structures, namely the correlation structure between one observation period and the next observation period and the correlation structure within outcome variables in a given observation period. Drs. Young Jack Lee and Taesung Park have been studying this problem. Preliminary data analyses show that the analysis is sensitive to the assumption of covariance structures. Drs. Lee and Park are looking into robust methods.

Missing covariates in logistic regression

In analyzing the DIEP study data, relations between blood chemistries and the fetal viable status at the end of the first trimester are of important medical interest. Blood samples were to be taken every week in the DIEP study, but many subjects were missing weekly visits and thus blood samples. Excluding subjects with missing blood samples from analyses will not only bias the statistical data analysis but also seriously reduce the size of analyzable data. One method of correcting this problem is to impute estimated missing covariates and analyze the augmented data. In estimating missing covariates, linear relations among covariates and the dependent outcome variable are assumed. Drs. Young Jack Lee and Haesook Kim have been studying a nonparametric method of estimating missing covariates by the local linear smoothing method. According to some preliminary evaluation, this nonparametric method of imputing missing covariates produces estimates for logistic regression parameters with smaller bias. An invited presentation was made at the Annual Statistics Meetings in Chicago.

A new decision rule in active control equivalence studies.

In active control equivalence studies, Drs. Kai Yu and Tie-hua Ng of the Food and Drug Administration have proposed a new decision rule based on the well-known attained significance level (p-value) and the attained Type II error probability (q-value) using either the conventional approach or the role-reversal approach. The p-value using the conventional approach is the same as the q-value using the role-reversal approach and the q-value using the conventional approach is the same as the p-value using the role-reversal approach. Consequently, the proposed decision rule does not depend on the formulation of the statistical hypotheses. Squabble over different formulation of the statistical hypotheses and the power consideration after the trials have been completed becomes unnecessary. Results have been published in the ASA Proceedings, Biopharmaceutical Section.

Multivariate permutation tests

Dr. Troendle has been working on the most powerful permutation test for a simple normal alternative in the multivariate two sample location shift problem. To test against composite alternatives requires the estimation of certain parameters, and thus no most powerful similar test exists in general. However, for two sided alternatives the natural estimates (ML) yield Hotelling's T^2 statistic, and for one sided alternatives a simple approximation yields a test very close to the likelihood ratio. A manuscript is in preparation.

Sequential testing with interval censored data

Drs. Kai Yu and Hui Quan of Merck, Sharp and Dohme, a former visiting fellow of the BMSB, have been collaborating on developing a sequential statistical test to test the mean levels of lifetimes which are interval-censored. The power and expected time for the decision are compared with those for the idealized situation when each and every actual lifetime is recorded. They are also compared with those for the case when one and only on inspection is allowed to make the decision. Results have been published in the Journal of Statistical Computation and Simulation.

Collaborative Research

The BMSB is actively providing statistical consulting support and collaborations to intramural scientists, DESPR and NICHD scientists. The BMSB staff are also working with outside scientists. Selected collaborative and consulting projects are described briefly.

Meta-analysis of trials of calcium for preeclampsia prevention

Dr. DerSimonian has analyzed, in collaboration with Dr. Richard Levine, the data available from previous trials of calcium for preeclampsia prevention. The goal was to describe the evidence and understand CPEP data in terms of results from other published trials. Part of this collaborative research was first presented to the final meeting of CPEP Steering Committee and as an invited talk (titled "Trials of Calcium for the Prevention of Preeclampsia") at the tenth World Congress: International Society for the Study of Hypertension in Pregnancy, Seattle (August 1996).

Vasectomy and prostate cancer risk

In collaboration with Dr. John Clemens, Dr. DerSimonian has reviewed the epidemiological data relating vasectomy to prostate cancer which indicates that any increase in risk of prostate cancer due to vasectomy is likely to be small or, at most, moderate. Furthermore, the methodological quality of the evidence reveals several weaknesses which make the interpretation of the results difficult. Namely, most, if not all, of these studies were subject to various biases that could have contributed to the increased risk observed in several individual studies as well as to the increased overall risk. Our research indicates that about 12% of the cases in the US will occur as a result of previous vasectomy even if a slight risk exists. In turn, this implies that about 38,000 new prostate cancer diagnoses a year could be attributable to vasectomy. New data from similar epidemiological studies on this topic, however, are unlikely to add to this understanding since epidemiology as a scientific discipline is limited in resolution when dealing with observational data.

Clinical Trial of Calcium for Preeclampsia Prevention (CPEP)

CPEP(Principal Investigator: Dr. Richard Levine) is a randomized, placebo-controlled, double-blind trial of calcium supplementation in US nulliparous pregnant women with gestational age at the start of therapy ranging from 13 to 21 weeks and expected average dietary calcium intake of about 1200mg/day. CPEP calcium supplementation (excluding calcium in prenatal vitamins) is set at 2000 mg/day. Subject recruitment for this five-center trial extended from May 1, 1992 to March 31, 1995, with a total sample size just exceeding 4500. Dr. DerSimonian has been involved with this project as the primary Institute statistician since 1990. Her responsibilities have included site visiting the clinical centers and data coordinating centers at the start of the project and actively participating in all subsequent stages of trial design and conduct; these include development of protocol, study forms, and manual of operations, supervision of all interim and final data analyses, and presentation of material at meetings; member of the Operating Committee, Steering Committee, and Data and Safety Monitoring Board. The data collection phase is now over and the final data analysis is in progress under Dr. DerSimonian's guidance.

Serum caffeine metabolites and pregnancy outcome

The relationship between maternal caffeine intake during pregnancy and the outcome of the pregnancy is controversial. Caffeine consumption has been found to be associated with increased risk of spontaneous abortion and reduced fetal growth, but these findings have been criticized as biased since ascertainment of caffeine exposure was from self report. This project consists of a pilot study and a main study. The pilot study includes samples from a validation population to determine whether a single serum level of caffeine, drawn at an unspecified time during the day, is useful to distinguish light from heavy caffeine users. The validation population includes women enrolled in the Birmingham (Alabama) fetal growth study. These women had both a 24-hour dietary recall and a serum collection at study entry which will be utilized to assess the relationship between caffeine intake and serum metabolite levels.

The main study will determine the association between maternal serum biomarkers of caffeine intake during pregnancy and the subsequent occurrence of spontaneous abortion, reduced fetal growth and adverse neuro-development in the offspring. This association will be measured among women registered in the Collaborative Perinatal Project (CPP), a prospective, multicenter cohort of approximately 55,000 pregnancies registered from 1959-1966. CPP did not collect any data on caffeine consumption during pregnancy but did collect serum for each woman at study registration and at several points thereafter till delivery. The main study will address the relationship of maternal serum metabolites with adverse pregnancy outcomes. Utilizing the results from the pilot study relating caffeine consumption to serum metabolite levels, the goal of the main study is intended to assess the association of maternal caffeine consumption and adverse outcomes of pregnancy. Dr. DerSimonian works with Dr. Mark Klebanoff, principal investigator of the project.

Unintended consequences of public cholesterol screening: false reassurance

In the last decade, National efforts have focused on reducing the prevalence of elevated blood cholesterol levels, a major risk factor for coronary heart disease. It is estimated that by 1990, about 29 million adults in the United States attended public screening for identification of elevated blood cholesterol. This population-based approach to reduce blood cholesterol levels through screening and follow-up for diagnosis and treatment of those with high levels seems to be effective. That is, data from the National Health and Nutrition Examination Survey shows that blood cholesterol levels in the United States have dropped significantly between 1988 and 1991 compared to the two preceding surveys conducted between 1971 to 1987. The purpose of this project, therefore, is not to assess whether screening is effective in reducing cholesterol levels, but to assess its adverse effects. For instance, do screening participants informed of a negative result perceive false reassurance from residual risks and continue or even adopt an unhealthy lifestyle? To date, no prospective study with a comparison group has reported on the impact of a negative test results on subsequent health behaviors.

Using data from the National Heart, Lung, and Blood Institute's Model System for Blood Cholesterol Screening Program, this project examines whether not referring subjects with borderline cholesterol levels for further medical evaluation has a false reassurance effect on their health behaviors and perceptions of health. Borderline cholesterol levels fall above the desirable levels but below the elevated levels required for referral. The data for this study suggest that relative to either the desirable or elevated group, the borderline group seems to have falsely reassured perception of health and exhibits a negative health behavior; for example, members of this group tend to decrease or not increase behavior promoting healthy diet or physical activities. Dr. DerSimonian has been analyzing the data, and Dr. Lois Maiman is the P.I.

Epidemiology of injuries in infants

In the United States, among children less than 15 years of age the highest fatal injury rates are found in those less than one year of age where injuries claim more than 1,000 lives per year. Several studies have shown maternal factors, such as maternal age and education, to be associated with injury death in this high risk age group. All such studies, however, have relied primarily on the limited amount of information available on death certificates and thus have been unable to fully examine the epidemiology of intentional or unintentional injuries in this age group. This project examines the association between maternal, sociodemographic and birth characteristics and injury death in infants less than one year old. Data from the National Center for Health Statistics linked infant birth/death certificate data sets for 1983-1987 will be used to identify these risk factors for infant injuries in the United States and the trends over time. Dr. DerSimonian works with Dr. Ruth Brenner for this data analysis project.

Classification Systems for Birth Weight

Dr. Troendle, in collaboration with Drs. Allen Herman, Kai Yu, and Cara Krulewitch, has completed a project on standardization of infant mortality rates in national comparisons. The method of standardization developed is an extension of the idea of relative birth weight developed by Allen Wilcox. The concept of relative birth weight is generalized to include gestational age because if the desire to understand the differences in different populations infant mortality in terms of differences in birth weight and gestational age. A paper has been submitted for publication. During the analysis for this project, a potentially disastrous scale conversion problem was discovered. The problem arises when a scale other than that with which the data was measured on is used to group the data, and manifests itself as a digit preference. The data to be grouped should always be grouped in intervals whose widths are a multiple of the lowest precision for which the values were measured. A short note has been accepted for publication in American Journal of Public Health.

Infectious Disease Death in Postneonatal Infants

Data from the 1983 to 1987 NCHS linked birth/infant death data sets were analyzed to identify factors associated with postneonatal mortality due to infectious diseases. The analysis revealed postneonatal deaths due to infectious disease were associated with the following factors: birth weight, preterm birth, African-American race, male gender, maternal education, maternal age, live birth order, number of prenatal visits. A paper has been accepted for publication in American Journal of Public Health. Dr. Troendle has been providing the statistical support. Drs. Jennifer Read and Mark Klebanoff are Dr. Troendle's medical counterpart for this data analysis.

Time to Treatment for Hypothyroidism in Cystinosis

In untreated cystinosis patients, thyroxine replacement for hypothyroidism is required in 75% of patients by the age of ten years. To determine whether early treatment with cysteamine prevents or delays the need for thyroxine replacement, 101 patients with cystinosis were followed. In the patients treated early, the average length of follow-up is significantly less than for patients treated late, making a comparison of the "survival" curves difficult. Proportional hazards regression models were used to show that early treatment with cysteamine does delay the need for thyroxine. Cysteamine therapy also had a positive impact on growth. A paper has appeared in Journal of Clinical Endocrinology and Metabolism. Dr. Troendle collaborated with Drs. Virginia Kimonis, T. Markello, and William Gahl of NIH, and analyzed the data.

Human Growth Hormone Trials

Dr. Troendle has been serving as the statistician preparing reports for the data safety and monitoring board for two NICHD growth hormone trials. Recently, the board asked Dr. Troendle to investigate the methods which would allow them to estimate the treatment effect for the primary outcome at this time which is complicated by the presence of dropouts and incomplete followup. This estimate would form the basis for a decision on whether to stop the recruitment or not. The methods explored include the EM algorithm and the repeated measures with missing data approaches.

Statistical Methods for Side-to-Side Difference in Nerve Conduction Studies

This study using theoretical derivations, statistical simulations and empirical estimations compares two ways of deriving reference values for side-to-side differences in nerve conduction parameters of normal subjects. The two methods involve using raw data of the side to side differences and the absolute values thereof. The theoretical derivations show that the population reference value of the absolute differences not equal to the true population reference value of interest. It is greater by 0.18%. In addition, the simulation studies show that the absolute difference yields greater sampling errors than the side to side difference method when establishing the reference values for nerve conduction parameters. The sampling variability is substantially reduced by using the sample size beyond fifty and becomes trivial as the sample size exceeds one hundred. Using H-reflex and extensor digitorium Brevis reflex clinical data, the two methods are compared. The absolute difference method overestimates the reference value by 0.4 ms for the extensor digitorium Brevis reflex, indicating an increase of Type II error. The side to side difference method is recommended in this study. Results are to be published in American Journal of Physical Medicine and Rehabilitation. Dr. Yu works with Dr. Audrey Chang of Walter Reed Army Medical Center) and Dr. Timothy Dillingham of Johns Hopkins University.

Preventing Problem Behavior Among Middle School Students

The goal of this study is to develop, implement, and test the efficacy of a multi-component program of school-based interventions in the primary prevention of problem behavior in a sample of middle school students. The study will be conducted in the Charles County, Maryland School District, which has seven middle schools with a student body that is 73% white and 25% black. Problem behaviors of interest include tobacco, alcohol, and other substance use; and conduct at school, home, and in the community. Objectives of the interventions are to increase students' social skills and self efficacy, facilitate conservative social norms, increase school bonding, and foster parental support and control. Interventions will be directed toward students to improve social skills and resistance to peer pressure; toward the school environment to encourage student bonding; and toward parents to foster support for student prosocial behavior. Students in three of the seven middle schools in the will be receive the special intervention that includes (1) social skills curriculum; (2) parent education; and (3) school environmental enhancement. Students in the other four middle schools will receive the enhanced school environment only. The primary research question is "What is the effect on the prevalence of problem behavior and their hypothesized mediators among students exposed to the special intervention compared to the comparison group?" Dr. Yu is the primary statistician for this project and works with Dr. Bruce Simons-Morton.

Intrauterine Growth Retardation, Preterm Birth and Long Term Neurodevelopmental Outcome

Two population based cohort studies were done to assess the impact of intrauterine growth retardation and preterm birth on neurodevelopmental outcomes at 5 years. The inception cohorts of 1518 indigent multiparous African American and white women who delivered at the University of Alabama at Birmingham, and 1552 para 1 and para 2 women who delivered at hospitals in Norway and Sweden (Uppsala, Bergen, Trondheim) from 1985 to 1988 were defined by 15 -17 weeks of gestation. Prenatal risk factors were collected at approximately 17, 25, 31 and 36 weeks of gestation. In both studies child cognitive development was assessed at age 5 with the Wechsler Preschool and Primary Scale of Intelligence - Revised (WPPSI-R) and neuromotor development was assessed using the Peabody Developmental Motor Scales. Approximately 25% of the two cohorts were lost to follow-up. IUGR and preterm children had significantly lower mean IQs at 5 years than term appropriate for gestational age children. The effects of IUGR and preterm birth held when certain postnatal factors were adjusted for (including maternal education, maternal reading level, the quality of the home environment, preschool attendance, parental income, maternal IQ, child rearing style, maternal social support and maternal psychological distress). Independent of IUGR and preterm birth, maternal IQ, the quality of the home environment and preschool education had the largest effects on IQ at 5 years. Dr. Yu has been analyzing the data, and Dr. Allen Herman is the P.I.

Maternally Linked Longitudinal Studies, Missouri and Utah

Dr. Kai Yu is the primary statistician for a series of data analysis projects of maternally linked longitudinal data of Missouri and Utah. Dr. Yu's collaborators are Dr. Allen Herman, Dr. Heinz Berendes, Dr. Cara Krulewitch (Center for Disease Control and Prevention), Dr. Yvette Johnson (Wayne State University), Dr. Jinrong Hou (National Institutes of Health) and Mr. Howard Hoffman (National Institutes of Health). A brief description of each project follows:

(1) Adolescent Age at First Pregnancy and Subsequent Obesity

Adolescent pregnancy has been associated with subsequent obesity. This study examines the patterns of obesity for second to fifth pregnancies among women who have their first singleton pregnancy as teenagers. Age, parity, interpregnancy interval and prior body mass index are significantly associated with subsequent obesity among adolescents. Race, marital status and smoking have significant interactions with age. Among older women being African-American and unmarried is associated with an increased risk of obesity, and smoking is associated with a decreased risk of obesity. Among younger women, there are no significant effects of race and marital status. The most important predictor of obesity is prior body mass index. This effect is present for both distant prior obesity and proximal prior obesity. First pregnancy body mass index has a greater effect on subsequent obesity if the intervening interpregnancy weight gains are large. Excessive weight gain during pregnancy presents the provider with a dilemma. An increase in birth weight associated with high prenatal weight gains may diminish the risk of infant mortality and morbidity in an index pregnancy, but subsequent obesity may increase perinatal mortality rates, the rates of obstetric problems and neural tube defects. Results are to be published in Pediatric and Perinatal Epidemiology.

(2) Different Fathers and Pregnancy Outcome

Preeclampsia (PE) is considered to occur mostly in primigravidae. However, studies have reported recurrence of PE when there is a change of paternity. It has been reported that each new father represents a similar risk in both multigravidae and primigravidae. Current research suggests that pregnancy maintenance is dependent upon an immune reaction that is initiated by paternal antigens. This immune reaction is hypothesized to be necessary for healthy placental development and infant growth. It has been suggested that PE is an immunologic response that is related to exposure to paternal antigens and that fetal loss and intrauterine growth retardation (IUGR) are due to poor placentation from an inadequate immune reaction. The purpose of this study is to examine the risk of poor pregnancy outcome (Preeclampsia, IUGR, Fetal Death) in sibships stratified by paternity. The Utah Successive Pregnancies Data set is a linked dataset containing information on women across their pregnancies. Information on the identity of the father is provided. A matched sibship case-control design stratified by paternity (different fathers within sibships and one father across sibships) was conducted to evaluate the risk of experiencing one of three poor outcomes. Sibships were matched on maternal age at first pregnancy, maternal race, sibship size, maternal and paternal education and occupation. Two controls were selected per case. Results are to be published in Paediatric and Perinatal Epidemiology.

(3) Siblings of SIDS Infants

The NICHD Sudden Infant Death Syndrome (SIDS) Cooperative Epidemiological Study showed that SIDS infants are at increased risk of intrauterine growth retardation (IUGR) compared to random Control A infants (rr=2.4; p<.01) and race and low birth weight-matched (within 250 g) Control B infants (rr=1.3; not significant). Recent studies in Norway suggest that siblings of SIDS are also growth retarded at birth. The authors have analyzed all 779 singleton SIDS (confirmed by autopsy) from the Missouri Successive Birth/Linked Death data set, 1978-1990. Four singleton Control A and B infants were randomly selected for each SIDS case. Control B infants were also matched to SIDS cases by size of sibship and birth order. There were 1448 SIDS siblings, 4691 Control A siblings and 5792 Control B siblings. Fetal and infant mortality rates (per 1000 births) were 9.7 and 16.7 for SIDS siblings, 6.4 and 11.6 for Control A siblings, and 11.2 and 19.9 for Control B siblings. Compared to Control B siblings, SIDS siblings were 1.55, 1.63, 1.87 and 2.43 times more likely to have mothers who were unmarried, less than 20 years old, not high school graduates, and smoking during pregnancy, all of which were statistically significant (p<.01). A lower mean birth weight (40 g; p<.05) was found for SIDS siblings compared to Control B siblings, after adjusting in a multiple regression for gestational age, sex, mother's age, weight, educational level, marital status, and race. However, after maternal smoking was added to the model, there was no longer a significant mean birth weight difference. Restricting to whites or smoking mothers resulted in SIDS siblings having slightly increased mean birth weight, which did not reach statistical significance. Thus, SIDS siblings were not at increased risk of IUGR when compared to Control B siblings (where the case and control B were matched for race and birth weight).

(4) Reproductive Compensation: A Reason for Intended Pregnancies among Adolescent Women

This analysis was carried out to assess the relationships between reproductive history and pregnancy outcome. Adolescent women of ages 11-19 years were compared to a reference group of women of 25 years. We used a unique data set of live births, fetal deaths, and death certificates for 1978-1990 from Missouri. The data was linked using a probabilistic linkage procedure and organized into sibship units. 432,502 linkages were attempted with an overall 85.5% success rate. Nine adolescent age cohorts (ages 11-19 years) were organized based on age at first pregnancy. The data were analyzed using Kaplan-Meier survival analysis for each age cohort as a function of interpregnancy interval(IPI) measured in days. Abortion rates (spontaneous and induced) were calculated for each age cohort. Results show: 1) For 12-14 year olds, the likelihood for second pregnancies in the year following the first are generally < 10%; this lower risk may be related to relatively low fecundity. 2) as age increases, this likelihood increases to 30% by age 19 years. 3) compared to the 25 year old reference group, adolescent women tend to have shortened IPI's (<1.5 years). This can be ascribed to high abortion rates in the youngest sexually active adolescents (75% in 13 year olds versus 15% in 19 year olds), as well as reproductive compensation in which women with a prior abortion or fetal death tend to replace their loss sooner than women with a previous live birth. We conclude: 1. reproductive compensation is one mechanism that leads to shortened IPI's due to prior loss. 2. shortened IPI's are more frequent among the young adolescent women. This may reflect a sociologic phenomenon, in addition to poor contraceptive choices among this group of women.

(5) Adolescent pregnancy, reproductive history and the risk of preterm birth and intrauterine growth retardation

It has been suggested that a deterioration in the general health of women in early adulthood is the major causal factor for the increased risk of poor pregnancy outcome. An alternative to this "weathering" hypothesis is that socioeconomic and behavioral risk factors accumulate with each successive pregnancy, especially among women who have their first pregnancies as adolescents. It is common practice to assess the relationships between reproductive history and pregnancy outcome using cross-sectional analytic approaches where women from different cohorts are grouped together. The authors used a unique Data set of live births and fetal and infant deaths for 1978-1990 from Missouri. The data was linked using a probabilistic linkage procedure to link all pregnancies of a woman. The births were arranged into sibship groups. 432,502 linkages were attempted with an 85.5% success rate. Nine adolescent age cohorts (ages 11-19 years) were organized based on age at first pregnancy. The risk of preterm birth and intrauterine growth retardation (IUGR) will be calculated using the following approach: two separate nonparametric logistic regression models will be used to compute the probability of preterm birth and IUGR among 25 year old women. Social and economic variables (e.g. education, marital status, pay status), behavioral risk factors (smoking, alcohol and drug abuse), interpregnancy interval and medical variables will be used as risk factors. The resultant regression coefficients will be then used together with the data from each pregnancy to calculate new predicted probabilities for poor outcome for women who have their first infants as adolescents. These predicted probabilities will then be used to examine the null hypothesis that women who have their first pregnancies as adolescents do not increase their risk level with each successive pregnancy.

(6) Maternal Attitude towards Weight Gain during Pregnancy and Adverse Pregnancy Outcome

Maternal attitude towards weight gain, especially for young teenage mothers, may lead to inadequately low weight gain during pregnancy and contribute to poor fetal growth. This question is addressed in the context of a much larger population based study of risk factors for adverse pregnancy outcomes in Missouri. A scale based on 21 questions probing maternal attitudes about weight gain during pregnancy were asked of mothers participating in the 1990 Missouri Maternal and Infant Health Survey. Surveys were available for 478 mothers of fetal deaths, 902 mothers of very low birth weight (VLBW, < 1500 grams), 802 mothers of moderately low birth weight (MLBW, 1500-2499 grams), and 920 mothers of normal birth weight (NBW, >= 2500 grams) infants. All mothers were Missouri residents when delivering their December 1989 to March 1991 births. A multivariate analysis procedure, principal components (PCS) analysis, was performed on a randomly selected one-third of the mothers to identify putative clusters of mothers with similar profiles of attitudes toward weight gain during pregnancy. The first 5 PCS, accounting for the largest percentage of variance, were independently derived and confirmed from the remaining two-thirds of the mothers, indicating stability for the clusters. One cluster identified generally negative attitudes about pregnancy, while another was generally positive about high weight gain and pregnancy. The remaining three clusters showed varying levels of concern about weight gain, with some mothers willing to gain more weight if it would improve the chances of a healthy baby. The relationship between mothers' attitudes towards weight gain and the health of the infant, and the association of maternal weight gain attitudes to other risk factors for low birth weight and/or preterm delivery will be explored while adjusting for race, educational level of the mother and household income.

Maternal Risk Factors for Intrauterine Growth Retardation and Preterm Labor and Delivery

This project, an NIH-DC initiative to reduce infant mortality, is a cohort study of maternal risks for intrauterine growth retardation and preterm labor and delivery in the District of Columbia. African-Americans have an almost two-fold risk of infant mortality compared to whites. The disparity is commonly attributed to social and economic conditions, increasingly psychosocial factors and adverse health behaviors such as smoking, alcohol and drug abuse are listed as reasons for excess mortality and morbidity. The examination of the impact of social, economic, psychosocial, cultural, and behavioral risk factors on intrauterine growth retardation and preterm labor and delivery may clarify the reasons for the excess in infant mortality among African-Americans. The study approaches the problem of low birth weight by specifying the outcome variables using clearly defined biological criteria. The risk factors of poverty, psychosocial stress, behavioral risk factors and prenatal care are defined. Potential biological consequences of these risk factors are described, and an attempt is made to bridge the social the social, economic and psychosocial factors through biological factors to poor pregnancy outcome. Dr. Kai Yu is the primary statistician for this project and Dr. Allen Herman is the P.I.

Better Babies Project in the District of Columbia

In support for Drs. Allen Herman and Heinz Berendes, Dr. Kai Yu performed data analysis for an enriched prenatal intervention program designed to reduce the risk of low birth weight. Free standing community-based prenatal intervention project is located in a poor inner city community, serving mostly African-American women. All women less than twenty weeks pregnant are eligible to participate. They are compared to women who live in neighborhoods with similar rates of poverty. The birth certificate is the source of data on maternal age, education, marital status, timing and frequency of prenatal attendance, parity, gravidity, prior pregnancy terminations, fetal and child deaths and birth weight. Thirty-eight percent of the women who delivered live-born infants in the study area participate in the program. There are no differences in low and very low birth weight rates in the study and comparison groups. In a secondary analysis comparing participants and non-participants in the study census tracts, participants are at higher risk for low and very low birth weight; they adhere more closely to the schedule of prenatal visits than nonparticipants. Low and very low birth weight rates are lower among participants than among non-participants and comparison women. The Better babies Project did not have an effect on the overall low and very low birth weight rates in the study census tracts. This is probably due to the low participation rates and the high population mobility. Results are to be published in Health Services Research.

Sperm Production in Testicles Obtained in Autopsy

The objective is to study whether sperm production has declined over time. We propose to perform a study of testicular wedge biopsies obtained at autopsy of men who died from unnatural causes. Blood serum will also be collected from each study subject by cardiopuncture at autopsy and stored at -70 degrees C. Specimens obtained from more than 130 men who died 10-20 years ago will be compared to those who die in the near future. The study is in the planning stage, and Dr. Yu works with Dr. Richard Levine.

Contraceptive development

Dr. Lee has been working with Drs. Pam Stratton and Adriane Fugh-Berman of Contraceptive Development Branch for contraceptive clinical trials. He participated in site visit for selecting a contractor for the statistical and clinical coordinating center for the clinical network for contraceptive research. At present two clinical trials are in the design stage. One is to test the CDB2914, an anti-progestin, against the Yutzpe preparation in preventing unwanted pregnancy after unprotected sexual intercourse. The other is to test the irritability and acceptability of the C31G spermicide in phase I setting. Dr. Lee has been designing both CDB-2914 emergency contraceptive and C31G spermicide studies. The CDB2914 study will be a phase II/III stage to determine if the CDB2914 causes a lower rate of vomiting and nauseating than the Yutzpe preparation and if the CDB2914 is as effective. The failure rate of Yutzpe preparation is less than 2%, where about 8% of unprotected sexual intercourse will result in pregnancy. As for the C31G spermicide, a comparative phase III clinical trial versus nonoxynol-9 will follow the phase I study.

ETEC vaccine study in Egypt

Dr. Lee works with Drs. John Clemens of DESPR and Stephen Savarino of Naval Medical Research to test ETEC vaccine in Egypt. Dr. Lee provides all statistical support including randomization, sample size determination, basic designs, and data analysis. At present, data on evaluating safety and immunogenicity of ETEC vaccine in children is being analyzed. A phase II study involving toddlers is in the design stage. Once the ETEC vaccine is found safe in toddlers, a phase III clinical trial of Egyptian infants will follow. A number of manuscript are in progress.

Cholera vaccine trial in Vietnam

Dr. Lee provides Dr. Clemens with statistical support for the cholera vaccine trial in Vietnam. Dr. Lee is the primary statistician for the trial. In this trial, each village is divided into two districts, residents of one district will be randomized to receive the cholera vaccine and residents of the other district to receive the placebo. About thirty villages will be thus randomized. The primary outcome variable is the number of cholera cases confirmed.

Diarrheal morbidity among infants of HIV infected mothers

The purpose of this project is to study epidemiology of enteric illness, nutritional function, and physical growth among infants born to HIV-infected women at the University of Maryland Hospital. Particularly, Drs. Haesook Kim and Lee are studying effects of HIV status on the risk of wasting. The data consist of 159 infants and their follow-ups. The data analysis is in progress. Drs. John Clemens and Karen Kotloff of the University of Maryland are P.I.'s of the project.

Data analysis support for DIEP study data

The BMSB is providing statistical data analysis support for the DIEP study. Drs. Eunsik Park and Young Jack Lee are analyzing the change of glucose level during the first trimester of normal pregnancy. There is a definite decreasing trend during the first trimester. Although there is a significant difference among centers in glucose levels, the rate of change of glucose levels does not vary from center to center. Drs. Park and Lee are focusing on whether the rate of decrease changes at some time during the first trimester. Dr. James Mills is preparing a manuscript on this analysis. Drs. Haesook Kim and Lee have analyzed the effect of glycosylated protein and fructosamine on fetal loss in normal pregnant women. There was a significant relationship. However, DIEP investigators concluded that the relationship could very well be an artifact of center-to-center variation of blood chemistries.

Neural tube defect studies in Ireland

Folic acid deficiency raises the risk of neural tube effect. A project is in progress in Ireland on what dose and duration it takes to raise the red-cell folate level to a protective level in women whose red cell folate level is not considered protective. A double blind clinical trial involving four dose groups is in progress in Ireland. Dr. Lee has been providing a statistical collaboration to Dr. James Mills.

Tubal ligation study data analysis

The Contraceptive Evaluation Branch of NICHD has evaluated two different types of tubal ligation, one cautery and the other clip, by a randomized clinical trial. The study also had a non-tubal ligation control group. The total size of the study is 180 women in reproductive age, 60 in each group. The study was to investigate the effect of tubal ligation on progesterone and estrogen during the luteal phase and to find out whether different types of tubal ligation would have different effect on production of reproductive hormones. Patients were followed up to four years post-surgery. These hormone questions have important medical implications, because endogenous progesterone and estrogen may be related to the ovarian cancer later in life. Dr. Lee is the primary statistician for the analysis of the data. The baseline data analysis is over, and two papers are in preparation where Dr. Lee is a co-author. The followup data analysis is in progress. The univariate analysis has been completed. Drs. Taesung Park and Lee are collaborating on the multivariate longitudinal analysis of the follow-up data.

Epidemiology Branch

The Epidemiology Branch of the Division of Epidemiology, Statistics, and Prevention Research, conducts research on epidemiological questions about medical disorders of pregnancy and childhood. Branch research staff include eight senior scientists, two post-doctoral fellows, one pre-doctoral fellow and two computer systems analysts.

Research conducted by Branch investigators currently focuses on perinatal issues, including disorders of pregnancy, low birth weight pregnancy outcomes, and congenital malformations; pediatric injuries; and pediatric infectious diseases, with a special emphasis on vaccine evaluation. Last year, the Branch was designated by the World Health Organization as an International Collaborating Centre for Vaccine Evaluation in Developing Countries.

Within these research domains, projects include randomized clinical trials of interventions and observational studies of risk factors. During the past year, the Branch's projects have been conducted at both domestic and international sites. The latter include projects in Bangladesh, Chile, Egypt, Ethiopia, India, Ireland, and Vietnam.

1. PREGNANCY

1.1 Calcium supplementation trial

The results of thirteen clinical trials and five meta-analyses suggest that supplemental calcium may prevent preeclampsia. Methodologic problems, however, and differences in study design limit the credibility of the results and their generalizability to other patient populations. Moreover, none of the trials has reported the outcome of systematic surveillance for urolithiasis, an important possible complication of treatment. In response to the need for a definitive evaluation of the effects of calcium supplementation, NICHD has conducted a trial at five U.S. university medical centers. Healthy nulliparous pregnant women were randomly assigned to receive either 2 g of daily ingested supplemental calcium (n = 2295) or placebo (n = 2294) in a double-blind study. Medication was administered beginning between 13 and 21 completed weeks of gestation and was continued until the termination of pregnancy. Monitoring for the major study endpoints - pregnancy-associated hypertension and proteinuria, preeclampsia, eclampsia, and HELLP syndrome - and for urolithiasis was systematic, standardized, and thorough. It included measurement of blood pressure, proteinuria, and hematuria at uniformly scheduled prenatal clinic visits and surveillance for hypertension and proteinuria during labor, delivery, and the first 24 hours postpartum. Data collection was completed during October 1995, and the data are currently being analyzed.

1.2 Diabetes in pregnancy

The Diabetes in Early Pregnancy (DIEP) Project continues to address risk factors for adverse pregnancy outcomes. The analysis of serum anticardiolipin and antiphospholipid antibodies has been completed. The prevalence of these antibodies was found not to be significantly greater in women who experienced spontaneous abortions than in women who had term deliveries. A manuscript describing the results has been submitted for publication. In a related study the presence of antisperm antibodies was compared in women who had spontaneous abortions and women who delivered at or near term. No excess was observed in Ig A, Ig M or Ig G antibodies among the women who miscarried. These findings have been published in Fertility and Sterility. It has been suggested that infection might cause early fetal loss. The availability of detailed data in the DIEP data base about infections of all kinds has allowed study investigators to pursue this question. No association was found with type and severity of infection. These results have been published in Human Reproduction. Currently in progress are analyses of changes in the insulin dose required by diabetic women as a result of their pregnancy and the influence of ketones on malformations and birth weight in children of diabetic women and in controls. Plans for future work include analyses of minor malformations and the influence of nutrition on early events in pregnancy.

1.3 Prostaglandin metabolism in preeclampsia

A disturbance of prostaglandin metabolism is currently believed to be one of the major pathophysiologic mechanisms underlying preeclampsia. Most previous studies of this theory, however, have included only women who had already developed clinically overt disease. In order to investigate whether prostaglandin abnormalities precede the onset of preeclampsia, Branch investigators are conducting a nested case-control study within the Calcium for Preeclampsia Prevention trial. Urinary excretion of selected prostaglandin metabolites will be compared in preeclampsia "cases" versus "controls" without preeclampsia, pregnancy-associated hypertension, or pregnancy-associated proteinuria. Systematic collection of urine from participants at various points during pregnancy will enable differentiation of abnormalities that precede the onset of preeclampsia from those that follow. Moreover, comparison of cases and controls who received placebo in the trial will enable assessment of the role of these abnormalities as determinants of the risk of preeclampsia. The trial has been completed recently and the last specimens will soon be sent for assay.

1.4 Maternal caffeine intake

Studies on the effect of maternal caffeine consumption on pregnancy outcomes have been conflicting. Several reports have demonstrated an increased risk of spontaneous abortion and reduced fetal growth among women consuming at least 300 milligrams of caffeine daily (equivalent to three cups of coffee), while others have failed to find these associations. However, no study has used a biomarker to measure caffeine intake. This is particularly important, as there are wide inter-individual differences in the rate of caffeine metabolism. Therefore, a given dose of caffeine may have a greater biologic effect in some women than others. In addition, several studies have noted that adverse effects of caffeine are restricted to women who also smoke. To investigate these issues, Branch investigators are studying the association between serum levels of caffeine and its primary metabolite and the occurrence of spontaneous abortion and reduced fetal growth.

In the pilot phase of the study, serum obtained from women who completed a 24-hour dietary recall including caffeine intake was analyzed for levels of caffeine and its metabolites. Reported intake of caffeine was found to correlate well with serum levels of both caffeine and paraxanthine, caffeine's primary metabolite. Particularly in women who smoked, paraxanthine correlated better than did caffeine. A nested case-control study to be performed using serum obtained from women enrolled in the Collaborative Perinatal Project is in preparation. Cases will be women who experienced a spontaneous abortion, while controls will be women who experienced a live birth. Caffeine and paraxanthine will be measured in serum obtained at comparable times during gestation in cases and controls to assess the association between levels of these substances and spontaneous abortion. In addition, among the controls, the associations between maternal levels of caffeine and paraxanthine and the occurrence of reduced fetal growth and childhood neurodevelopmental disability will be studied. The pilot data are being prepared for publication, and a laboratory to analyze serum for the case-control study is currently being sought.

1.5 Reproductive compensation in adolescence

Numerous reports have demonstrated that adolescent mothers have an increased risk of stillbirths, premature births, abortions, and perinatal deaths. One factor that may increase the risk of adverse pregnancy outcomes in adolescent mothers is shortened birth spacing. Reproductive compensation is a phenomenon in which women with a prior pregnancy loss tend to replace that loss sooner; this results in shortened interpregnancy intervals between successive pregnancies. Branch investigators designed a unique population-based analysis to evaluate the relationship between previous pregnancy loss (abortion, perinatal death) and time to subsequent pregnancy in adolescent women. The Missouri Linked Data Set of live births, late fetal and infant deaths from 1978-1990 was used for this analysis. Results revealed high rates of abortion, perinatal death, preterm births, and small-for-gestational age (SGA) births among adolescent women, especially the youngest adolescents. Overall, African American women had higher rates of preterm and SGA births. Adolescent women were more likely to have shortened interpregnancy intervals (<1.5 years) between successive pregnancies compared to the reference group; and African American women were more likely to have shortened interpregnancy intervals compared to white women (50% vs. 40%). These results suggest that reproductive compensation appears to exist in adolescent women, resulting in shorter interpregnancy intervals, and may partly explain the increased risk of perinatal morbidity and mortality seen in this age group. These findings were presented at the International Symposium on Maternally Linked Pregnancy Outcomes in Atlanta.

1.6 Contextual poverty, individual socioeconomic status and poor pregnancy outcomes in the District of Columbia

Contextual socioeconomic effects are potentially important causal factors for poor pregnancy outcome. Branch investigators examined the impact of contextual poverty, conditioned on maternal educational attainment, on low and very low birth weight in the District of Columbia. Contextual poverty was measured as the proportion of families in a census tract who live below the federal poverty line. Increasing contextual poverty was associated with increased risk for low and very low birth weight. Although most women with a college education lived in neighborhoods with low rates of poverty, at least 20% of college educated women lived in D.C. neighborhoods with more than 30% poverty. African American women who had not graduated from high school, but who lived in the least poor neighborhoods, had rates of low birth weight similar to those of college-educated women who lived in those neighborhoods. College-educated women who lived in the most impoverished neighborhoods had rates of low birth weight similar to those of women without a high school education. The contextual effects of socioeconomic status thus appeared to be independent of the impact of individual social status variables.

1.7 Impact of changing paternity on pregnancy outcomes

Preeclampsia (PE) is considered to occur mostly in primigravidas. However, studies have reported recurrence of PE when there is a change in paternity. It has been reported that each new father represents a similar risk in both multigravidae and primigravidae. Current research suggests that pregnancy maintenance is dependent upon an immune reaction that is initiated by paternal antigens. This immune reaction is hypothesized to be necessary for healthy placental development and infant growth. It has been suggested that PE is an immunologic response that is related to exposure to paternal antigens and that fetal loss and intrauterine growth retardation (IUGR) are due to poor placentation from an inadequate immune reaction. Branch investigators examined the risk of recurrence of PE, IUGR, and fetal death in sibships stratified by paternity. This analysis used the Utah Successive Pregnancies Dataset, which is a linked dataset containing information on women across their pregnancies. Information on the identity of the father is provided. Among women who had an intrauterine growth retarded infant in an earlier pregnancy, a change in paternity did not result in an increased risk of recurrent IUGR. Among women with no poor outcome in an earlier pregnancy, there was a twofold increase in subsequent IUGR with a change in paternity. Half of this increase appeared to be due to socioeconomic changes that took place with a change in paternity. The paper is to be published in a special issue of **Paediatric and Perinatal Epidemiology**.

1.8 Very preterm twin birth: role of race and fetal sex

Branch investigators previously found that before 34 weeks gestation male births exceeded female births, but that this preponderance of male births did not occur at later gestations among white New England twins. This suggests the existence of a mechanism of very preterm twin birth governed by fetal sex. The Missouri Sibship data set enables an examination of linked twin pairs. Among 3297 twin births in whites, 21% of male:male twins were born at <34 weeks gestation, in contrast to 15% of sex discordant twins (P<.001 vs. male: male births) and 17% of female: female twins (P<.01 vs male: male twin births). Having a male fetus was not associated with an increased risk of very preterm birth when a female twin was present. These results suggest a sex-linked recessive disorder causing very preterm birth, or protection from preterm birth afforded by the female fetus. Position of the male fetus did not effect the risk of very preterm birth.

1.9 Adolescent age at first pregnancy and subsequent obesity

Adolescent pregnancy has been associated with obesity later in life. Branch investigators examined the patterns of obesity for second and third pregnancies among women who had their first singleton pregnancy as teenagers, using maternally linked data from 1978 through 1990 among 43,160 Missouri resident women. Age, parity, interpregnancy interval and prior body mass index were significantly associated with subsequent obesity among adolescents. Race, marital status and smoking had significant interactions with age. Among women with pregnancies at older ages, being African-American and never married was associated with an increased probability of being obese, and smoking had a greater effect on obesity at higher maternal age. Race and marital status did not have significant effects on obesity among younger pregnant women. The most important predictor of obesity was prior body mass index. Body mass index before the first pregnancy had a greater effect on subsequent obesity if the intervening interpregnancy weight gains were large. Excessive weight gain during pregnancy presents the provider with a dilemma. An increase in birth weight associated with high prenatal weight gains may diminish the risk of infant mortality and morbidity in an index pregnancy, but subsequent obesity may increase perinatal mortality rates, the rates of obstetric problems and neural tube defects. This paper is in press in a special issue of **Pediatric and Perinatal Epidemiology.**

1.10 Evaluation of the effectiveness of a community-based, enriched model prenatal intervention project (Better Babies Project) in the District of Columbia

Branch investigators evaluated an enriched prenatal intervention program designed to reduce the risk of low birth weight in a poor inner city community, serving mostly African American women. All women less than 29 weeks pregnant were eligible to participate. They were compared to women who lived in neighborhoods with similar rates of poverty. The birth certificate was the source of data on maternal age, education, marital status, timing and frequency of prenatal care attendance, parity, gravidity, prior pregnancy terminations, fetal and child deaths and birth weight. Thirty-eight percent of the women who delivered live-born infants in the study area participated in the program. There were no differences in low and very low birth weight rates in the study and comparison groups. In a secondary analysis comparing participants and nonparticipants in the intervention census tracts, participants had higher risk profiles for low and very low birth weight outcomes than non-participants , and they adhered more closely to the schedule of prenatal visits. However, low and very low birth weight rates were lower among participants than among nonparticipants and comparison women. The Better Babies Project did not have an effect on the overall low and very low birth weight rates in the study census tracts. This was probably due to the low participation rates and the high population mobility. This paper is in press in **Health Services Research.**

2. LOW BIRTH WEIGHT

2.1 Intergenerational studies

It is known that low birth weight tends to recur across generations. Evidence from linked birth certificates of mothers and children born in Tennessee indicated that the correlation was stronger for the rate of intrauterine growth of the mother and the fetus than for the length of gestation of the mother and the fetus. In an additional study by the Branch, investigators studied the relationship between a mother having been born small-for-gestational age (SGA) or preterm and giving birth to a SGA or preterm infant. 1298 women born in Denmark between 1959-61 and their 2046 singleton infants were analyzed. The study was unique because information on paternal birth weight and adult weight was also available for analysis. The data revealed that SGA mothers were more likely to give birth to SGA, but not preterm, infants; and, conversely, that preterm mothers were more likely to give birth to preterm, but not SGA, infants. Paternal birth weight was also directly associated with infant birth weight, but to a lesser degree than maternal birth weight. These findings demonstrate the growth-restraining effects of both parents on the fetus and underscore the need to account for parental birth weight in studies of low birth weight. The results were presented at the 1996 annual meeting of the Society for Pediatric Epidemiologic Research.

Branch investigators have recently completed a similar study in which women who were born in 1959-66 in the Philadelphia and Providence cohorts of the Collaborative Perinatal Project were located and the outcomes of their pregnancies determined. Analysis of this study is in progress.

2.2 Effect of maternal diet and nutritional status upon birth weight in a less-developed country

Maternal undernutrition is thought to underlie most of the fetal growth retardation observed in developing countries. In Ethiopia, where the diet is commonly deficient in calories, pregnant women show varying degrees of undernutrition that correlates with the level of calories in the diet. Branch investigators estimated the household food energy consumption of 136 pregnant women from a 4-day weighed dietary survey. Using the FAO/WHO/UNU criteria for assessment of adequacy of dietary energy intake, 70% of households had levels of dietary intake that would allow only the minimum of physical activity. Women of these households and their spouses had significantly reduced body mass index compared to those with adequate intake. However, the occurrence of low birth weight babies in these pregnancies was low (4%), and the difference in mean birth weight between those with low and adequate energy intake was only 100 g. The apparent lack of correlation of the home diet with birth weight indicates that, at least in this population, there appear to be factors other than diet that tend to protect the fetus from deficiencies in food supply.

2.3 Surrogate measures of low birth weight in a less-developed country

In developing countries it is often difficult to obtain accurate measurements of birth weight and gestational age, because women have limited access to prenatal care and because most babies are born at home and may not be seen by a health care provider until several days or even weeks after birth. In an earlier study of nearly 1000 Ethiopian newborns, Branch staff demonstrated that a simple algorithm that incorporated chest and head circumferences measured at birth accurately identified preterm, low birth weight babies. However, for the algorithm to be useful in the field, the measurements upon which it is based--chest and head circumference-- must be stable, at least during the first week of life. In a recent follow-up study, Branch investigators assembled a group of 330 newborns from the obstetric service of the hospital of the University of Addis Ababa and measured body weight as well as head, chest, and mid-arm circumferences on days 1, 3, and 7 of life. As expected, weight declined during the first week, by 5% at 3 days and 3% at 7 days. Interestingly, mid-arm circumference values also declined by 5% and 7% at days 3 and 7. In contrast, chest and head circumference measurements were stable, and the sensitivity and specificity of the original algorithm for identifying preterm, low birth weight babies showed almost no change between days 1, 3, and 7 of life. These findings confirm the utility of the algorithm during the first week of life, and provide the basis for field-testing of the algorithm in a public health program designed to identify and treat premature, low birth weight neonates.

2.4 Birth weight related to growth and health by age six

The third National Health and Nutrition Examination Survey (NHANESIII) has collected descriptive data, physical measures, and biomedical samples on a representative U.S. sample of children at ages two months through six years with oversampling for blacks and Mexican Americans. Branch investigators have linked the extensive data from NHANESIII to birth certificates. The following analyses are in progress: 1) determination of the association between low birth weight or prematurity and subsequent poor health as measured by biomedical markers in a representative national sample of children through age six; 2) assessment of the effects of ethnic/racial and socioeconomic factors on these outcomes; 3) development of normative growth and other biomedical scales for a U.S. representative population, and for black and Mexican-American children, according to a risk profile at birth; 4) comparisons between maternal and proxy reports of children's risk factors at birth and actual measurements; and 5) determination of effects of environmental tobacco smoke on the growth and health of young children.

2.5 Survival and health of high-risk deliveries

Branch investigators are determining the effect of the available level of technological support at delivery on live birth status, survival to age one and subsequent health status at age three for premature infants. Data from a nationally representative sample of births and fetal deaths occurring in 1988 for which survivors were followed until age three have been linked to information on the technological capacity of the delivery hospitals, obtained from the American Hospital Association. Additional information on hospital participation in experimental surfactant protocols has been obtained. Collaborators include staff of the National Center for Health Statistics, National Perinatal Information Center, and the March of Dimes.

2.6 Low birth weight and infectious disease mortality

Using the 1987 linked birth-death cohort files from the National Center for Health Statistics, Branch investigators studied factors associated with post-neonatal death due to infectious diseases. After adjusting for confounding factors, children who weighed less than 1500 grams were over 26 times, and children who weighed 1500 to 2499 grams were 3.5 times more likely to suffer a post-neonatal death due to infectious disease than were children with birth weights of at least 2500 grams. A manuscript presenting these results is in press in the American Journal of Public Health.

2.7 Intrauterine growth retardation, preterm birth and long-term neurodevelopmental outcomes

Branch investigators conducted two population-based cohort studies to assess the impact of intrauterine growth retardation (IUGR) and preterm birth on neurodevelopmental outcomes at 5 years. The inception cohorts of 1518 indigent multiparous African American and white women who delivered at the University of Alabama at Birmingham, and 1552 para 1 and para 2 women who delivered at hospitals in Norway and Sweden (Uppsala, Bergen, Trondheim) from 1985 to 1988 were assembled at 15 - 17 weeks gestation. Prenatal risk factors were collected at approximately 17, 25, 31 and 36 weeks of gestation. In both studies child cognitive development was assessed at age 5 years with the Wechsler Preschool and Primary Scale of Intelligence - Revised (WPPSI-R), and neuromotor development was assessed using the Peabody Developmental Motor Scales. Approximately 25% of the two cohorts were lost to follow-up. Compared with children born at term who had birth weights appropriate for gestational age, IUGR children and children born preterm had significantly lower mean IQs at 5 years. The effects of IUGR and preterm birth held after adjustment for maternal education, maternal reading level, the quality of the home environment, preschool attendance, parental income, maternal IQ, child rearing style, maternal social support and maternal psychological distress. Independent of IUGR and preterm birth, maternal IQ, the quality of the home environment and preschool education had strong associations with IQ at 5 years.

3. BIRTH DEFECTS

3.1 Irish neural tube defects study

Branch investigators continue to work with collaborators at Trinity College, Dublin and the Health Research Board of Ireland to examine the mechanism by which folate reduces the risk of neural tube defects (NTDs). Genetic abnormalities have been sought that could account for the elevated homocysteine levels previously discovered by Branch investigators in pregnancies complicated by NTDs. During the past year simultaneous reports by Branch investigators and collaborators and by investigators from the Netherlands have identified a genetic cause of elevated homocysteine. The abnormal gene, which is observed more frequently in persons with NTDs, produces a thermolabile variant of the reductase enzyme. A search is underway for other gene abnormalities in enzymes related to homocysteine metabolism. Branch investigators are collaborating with Dr. Larry Brody of the NIH Human Genome Center. Dr. Brody has cloned the gene for methionine synthase and will look for polymorphic alleles to test for associations with NTDs.

Studies in female employees of a Dublin hospital will determine the dose of folic acid required to raise red cell folate to a level known to protect against NTDs. Other studies will examine the biochemical characteristics of women with a normal pregnancy who have had a past pregnancy with an NTD. Further biochemical and genetic investigations will be performed using placental material and fibroblasts obtained from NTD cases.

3.2 Fetal alcohol syndrome studies

A search for biochemical markers to identify women at risk for producing newborns with the fetal alcohol syndrome is under way at two prenatal clinics in Santiago, Chile. Women who are heavy drinkers are identified at their first prenatal visit by interview and subsequent home visit. Their pregnancies are monitored in follow-up interviews. Blood samples are obtained at three time points. The same data are collected on a control group of similar women without evidence of alcohol use. Babies of mothers who are heavy drinkers and of control mothers are examined at birth and subsequently to diagnose cases of fetal alcohol syndrome. Blood samples will be analyzed to identify markers of alcoholism which can be used to detect women at risk of bearing a child with fetal alcohol syndrome.

4. CHILDHOOD CANCER

4.1 Exposure to Maternal Smoking and Childhood Cancer

Cigarette smoking is the most common known cause of cancer in adults, and among the most preventable. In addition, maternal cigarette smoking during pregnancy is the largest preventable cause of low birth weight and a significant preventable cause of perinatal mortality. However, the degree to which maternal smoking during pregnancy influences the risk that her newborn will subsequently develop cancer during childhood is not known. In this study, Branch Investigators analyzed the association between maternal smoking during pregnancy and the subsequent development of cancer in the child using data from the Collaborative Perinatal Project. Children of women who smoked during pregnancy were 1/3 less likely to develop cancer than children of women who smoked. These results suggest that maternal smoking is not a cause of childhood cancer. These results are currently in press at the American Journal of Epidemiology.

4.2 Long-term outcomes of leukemia survivors

In collaboration with the National Cancer Institute, Branch investigators are evaluating outcomes of long-term survivors of childhood leukemia. This study group has recently completed a report to be published in Cancer on birth defects in the offspring of men and women who survived childhood leukemia. There was no increased risk in offspring of fathers or mothers who had had leukemia.

5. CHILDHOOD INJURIES

5.1 Drowning

Drowning is a leading cause of injury-related mortality among children. The Year 2000 Health Objectives of the U.S. Public Health Service call for a reduction of drowning rates among all age groups, and emphasize targeted interventions for children four years of age and under. In order to develop these targeted interventions information is needed on the circumstances surrounding the submersion event and on the specific site of the occurrence. This information is not available from national datasets but can be obtained from the free text portion of the death certificate. In collaboration with the Consumer Product Safety Commission, Branch investigators are abstracting information from death certificates for all non-boat related drowning deaths in children and adolescents. Results from this study will provide the first in-depth, national analysis of the circumstances surrounding drowning deaths.

5.2 Childhood injuries in the District of Columbia

As part of the NIH/DC Initiative to Reduce Infant Mortality, Branch investigators have developed a research program aimed at developing and evaluating interventions to prevent childhood injuries in the District of Columbia. Currently in the United States, there is no active surveillance system that provides epidemiologic data on nonfatal injuries at either the national, state or city level. The goal of the first phase of this study is to establish a city-wide surveillance system in the District of Columbia. Information on injuries occurring before the third birthday is being abstracted from death certificates, inpatient records and emergency room records. In addition, at three of the study hospitals the parent or primary caretaker is interviewed to provide further details on the circumstances of the injury. Information on substantiated cases of abuse and/or neglect is being abstracted from records at the Department of Family Services. The epidemiologic information gathered in this first phase will be used to guide the design of interventions aimed at preventing infant and early childhood injuries, to be tested in the second phase of the study.

5.3 Medically attended Nonfatal injuries in U.S. children and adolescents

Branch investigators coordinated the 1988 Child Health Supplement to the National Health Interview Survey. Information on nonfatal injuries in U.S. children and adolescents was included in the supplementary survey for the first time . A number of analyses of nonfatal injuries have been completed based on survey data. Reports of the findings have been published or are currently in press in the American Journal of Public Health, Archives of Pediatric and Adolescent Medicine, American Journal of Preventive Medicine, and Proceedings of the International Collaborative Effort on Injury.

The causes of non-fatal injuries were different from the causes of fatal injuries. For most non-fatal injuries the rates for males exceeded those for females at all ages. Rates for both sexes were highest during the preschool and adolescent years. Parents of children without health insurance or Medicaid were less likely to report medically attended injuries, regardless of injury severity. This suggests that studies based on injury reports from treatment sources could be biased when assessing the effects of risk factors correlated with lack of medical coverage. Sports and recreation accounted for 36 percent of all nonfatal injuries in children and adolescents.

Additional analyses showed that after controlling for maternal education, number of children, and race or ethnicity, children of single adult households had a 40 percent increased risk of injury. Analysis of injuries and child care demonstrated that increasing hours of non-guardian child care was not associated with increased risk of injury. However, children who were cared for in multiple locations were more likely to suffer injuries, regardless of severity.

In another national survey, Branch and other investigators found that almost 25 percent of children suffered medically-attended injuries during the first three years of life with 25% of these having more than one such injury.
Falls, burns, poisonings, and being struck or cut, were the most prevalent causes of non-fatal injuries.

5.4 Occupational injuries in U.S. children and adolescents

While about 4 million children and adolescents are legally employed in the U.S., an unknown number are illegally employed in violation of state and federal labor laws, with an estimated 24% of high school students working "off the books." Branch investigators collaborated on the Survey of Income and Program Participation of the U.S. Bureau of the Census. This survey will enable analyses of the determinants of the time worked by adolescents and the determinants and health consequences of their nonfatal occupational injuries. Preliminary analyses indicate that during the school year working 15-and 16-year old children are employed an average of more than 20 hours per week. Many young teens are working more hours per week than allowed by child labor laws. Analysis of injuries in these adolescents is underway.

5.5 International comparisons of injuries in children

Branch investigators are collaborating on an international research project on fatal and nonfatal injuries. The research focuses on strategies to improve the quality and reliability of international statistics. A paper by Branch researchers on population-based surveys as sources of U.S. injury data has been published in the Proceedings of the International Collaborative Effort on Injury Statistics. Analysis of risks for drowning among selected countries have demonstrated that many of these deaths are not reported due to defective mechanisms for verifying the cause of death. A survey of comparable injury data sources is currently being conducted to allow similar analyses on other topics.

In another international collaboration, Branch and other NICHD staff and the U.S. Substance Abuse and Mental Health Services Administration are participating in the WHO Study of Health Behaviors in School-aged Children. The goal of this study is to allow comparisons of risk factors for injuries in several countries in order to assess risks associated with cultural and behavior factors. A survey of U.S. children was completed during 1996, and preliminary comparisons of the U.S. data to findings from 27 other countries has begun.

5.6 Injury fatalities in infants

Injuries are the third leading cause of postneonatal death and perhaps the leading cause of preventable postneonatal death. Branch investigators are analyzing linked birth and death records for U.S. infants to assess the demographic, socioeconomic and prenatal risk factors for injury deaths during infancy. Results indicate that, of the variables considered (sex, race, maternal education, marital status, prenatal care, birth order, maternal age, birth weight, and gestational age), low maternal education is most strongly associated with injury death.

Homicides were the leading cause of infant injury death with 2,276 deaths from 1983-1991. Moreover, analysis of infant injury deaths that are classified as being of "undetermined intent" revealed risk factor profiles that more closely resembled intentional than unintentional injuries. These findings suggest that fatality rates from intentional injuries could be 10-15% greater than previously recognized. Factors most strongly associated with intentional infant deaths and with infant deaths of undetermined intent were maternal age <17 years, late or no prenatal care, and having a second or third child by age 19. Interventions to prevent intentional infant deaths should, therefore, target pregnancies in adolescent girls.

6. INFECTIOUS DISEASE EPIDEMIOLOGY AND VACCINOLOGY

6.1 Group B streptococcal infections

6.1.1 Protective level of maternal antibody against early-onset Group B streptococcal sepsis in neonates

Group B streptococcus (GBS) is the leading cause of neonatal sepsis and meningitis in the U.S. One strategy to prevent neonatal GBS disease would be to vaccinate women of child-bearing age, to elicit maternal antibodies that could be transferred across the placenta, thereby protecting the newborn. Current vaccines under development are based on the notions that GBS capsular polysaccharide (serotypes Ia, Ib, II, III, IV, V) is a major virulence factor of this organism and that protective immunity is serotype-specific. However, the titer of maternal serum antibodies at term that are protective against early-onset disease of the newborn is unknown. It is therefore not clear what titer of antibodies future vaccines should aim to elicit.

Branch investigators are conducting a multicenter case-control study to evaluate, for each GBS serotype, the pattern of the protective association between naturally acquired, serotype-specific maternal serum IgG antibody titers at term and the risk of early-onset GBS disease due to homologous serotypes in newborns. The study will assemble 140 early-onset GBS cases and 1120 surface-colonized but healthy newborn controls in the 13 participating hospitals of 6 U.S. academic institutions. Maternal blood at delivery and cord blood are collected from maternal-newborn dyads for each case and control. Serological assays of maternal and cord antibodies to each GBS serotype, which are currently being standardized at the Center for Biologics Evaluation and Research of the Food and Drug Administration, will be performed after the completion of surveillance. Assembly of cases and controls began in July 1995. During the first 12 months of surveillance, 51 early-onset GBS cases have been identified with an incidence rate of 1.23/1000 live births, and 605 colonized infants have been assembled with an infant colonization rate of 12.1%.

6.1.2 Group B streptococcal colonization during pregnancy

The Vaginal Infections and Prematurity (VIP) Study has provided a unique opportunity to evaluate several cogent issues regarding the epidemiology of Group B streptococcal (GBS) cervicovaginal colonization during pregnancy. Branch investigators addressed whether GBS colonization at 23-26 weeks' gestation is associated with adverse pregnancy outcomes, and whether treatment of colonized women with erythromycin improves pregnancy outcomes. Although these analyses demonstrated that colonized women were not at increased risk for preterm or low birth weight deliveries, or for rupture of membranes before labor, the subgroup of heavily colonized women who had not received antibiotics after mid-gestation were at higher risk for these outcomes. These findings, which were published in *The American Journal of Obstetrics and Gynecology*, raise the possibility that heavy colonization, rather than colonization *per se*, may be a risk factor for adverse pregnancy outcomes. However, in the clinical trial component of VIP, erythromycin therapy did not result in a reduction in preterm births or low birth weight infants among colonized women, even though the drug did effectively reduce the occurrence of colonization. Results of the trial were also published in The American Journal of Obstetrics and Gynecology.

6.1.3 Effectiveness of intrapartum antibiotic prophylaxis against early-onset neonatal group B streptococcal disease

Neonatal group B streptococcal (GBS) disease is the most common infectious disease affecting newborn infants with an estimated incidence rate of 1.8 per 1000 live births. The case-fatality rate of neonatal GBS disease has been reported to be 10-20% in infants, and severe neurologic sequelae have been noted in up to 30% of GBS meningitis survivors. Because of the public health importance of this infection, intrapartum antibiotic prophylaxis has been aggressively promoted for pregnant women at high risk of having a newborn with GBS. Although this strategy has been shown to be efficacious in one small clinical trial, the practical effectiveness of the strategy when implemented in routine clinical practice is unknown. Branch investigators have recently initiated a multicenter, retrospective case-control study of the effectiveness of intrapartum antibiotic prophylaxis in preventing early-onset GBS disease in infants born to women with one or more of the published indications for prophylaxis. **The study will evaluate 110 cases of early onset cases and 220 matched controls.** These cases and controls will be assembled from infants born between January 1, 1992 through December 31, 1994 at 11 U.S. hospitals.

6.2 Gestational syphilis

The rapid plasma reagin (RPR) test has been proposed as a simple but suitable test for detecting maternal syphilis infection in less-developed settings. Branch investigators evaluated the effectiveness of a program of screening of pregnant women with this test during prenatal care administered in Addis Ababa, Ethiopia. 3484 consecutive women presenting for care were tested by RPR, and all 580 who tested positive were treated with 2.4 million units of benzathine penicillin during the same clinic visit. Subsequent testing of stored sera permitted evaluation of the program in relation to confirmed syphilis status, as ascertained with VDRL and fluorescent treponemal absorption (FTA) tests. Overall, the RPR test had a sensitivity of 66% and a specificity of 89% in identifying confirmed positives and negatives, respectively. Penicillin therapy of women who tested positive by RPR and were confirmed by VDRL/FTA was associated with a reduced risk of late fetal and early (first 12 weeks) infant mortality (8%), compared with that (13%) observed for offspring of syphilitic women who falsely tested negative by RPR and were thereby not treated. However, the reduction of the risk of these outcomes in penicillin-treated women did not reach the level for offspring of women who did not have syphilis infections (3%). Although the risk of late fetal and early infant mortality was higher for women who were HIV-infected than for those who were not, the same gradients of risk by syphilis and penicillin status were observed for both the HIV-infected and non-HIV-infected groups. These findings demonstrate significant weaknesses in the RPR-testing and penicillin treatment strategy, despite its logistical advantages.

6.3 Cholera

6.3.1 Bangladesh trial of killed oral cholera vaccines

Branch investigators continued to conduct analyses of the 1985 field trial of B subunit-killed whole cell (BS-WC) and killed whole cell only (WC) oral cholera vaccines, which was conducted in the Matlab field area of the International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B). In a final analysis of the five years of follow-up of the approximately 63,000 children and adult women who received three doses of one of the vaccines or placebo in this trial, there were 144 cholera cases in the BS-WC group (Protective efficacy (PE)= 49%; P<.001), 150 in the WC group (PE= 47%; P<.001), and 283 in the K12 placebo group. Protection by each vaccine was evident only during the first three years of follow-up. Analyses of all vaccinees in aggregate revealed moderate (PE= 40-56%) vaccine protection against classical cholera during each of the first two years of follow-up of vaccinees aged 2-5 yrs, but only evanescent protection against El Tor cholera in this age group. Among older vaccinees, protection against classical cholera was more substantial and was sustained (PE=58-76%) during each of the initial three years; somewhat lower protection was maintained during this period against El Tor cholera (PE= 48%-64%). These findings, which were published in *Vaccine*, indicate that each vaccine conferred a similar level of protection against cholera over a five-year period of observation; that long-term vaccine protection of young children was observed only against classical cholera; and that the vaccines conferred three-year protection against both biotypes among older persons, but at a higher level against classical cholera.

In an additional analysis of the Bangladesh trial, Branch investigators evaluated whether vaccinated subjects who failed to be protected by BS-WC or WC vaccines exhibited deficient immune responses when they became infected by *V. cholerae* 01. During the first year of the trial, serum vibriocidal (VC) antibody responses to *V. cholerae* 01 infections were compared in three-dose recipients of BS-WC or WC versus three-dose recipients of an *E. coli* K12 placebo (K12). For persons dosed at ≥5 years of age, the age-group in which the vaccines were notably protective, geometric mean-fold (GMF) rises of VC antibodies were lower after 26 clinically severe cholera episodes in vaccinees than after 44 clinically severe episodes in K12 recipients (6.5-fold vs. 18.6-fold, P<.01). No response of serum VC antibodies was evident after the 16 asymptomatic infections in vaccinees (1.1 GMF-rise), in contrast to the 5.9 GMF-rise after 14 such infections in K12 recipients (P<.01). These results, which were published in *The American Journal of Epidemiology*, demonstrate that vaccinees who failed to be protected by efficacious vaccines exhibited poorer immune responses to clinically severe cholera, and notably deficient responses to asymptomatic infections. Since the immune stimulus delivered by asymptomatic infections is likely to resemble that elicited by suitably safe, live-attenuated oral vaccines, these data suggest that immune hyporesponsiveness may impose an upper limit to the efficacy attainable by vaccination of populations in which cholera is endemic.

6.3.2 Trial of killed oral cholera vaccine in Vietnam

Based on the encouraging results for killed oral cholera vaccines in the Bangladesh trial, described above, the National Institute of Hygiene and Epidemiology (NIHE) in Hanoi embarked on a program of local production of oral killed whole cell (WC) cholera vaccine. A study of this vaccine in Vietnamese adults in the late 1980s showed it to be both safe and immunogenic. Accordingly, in 1992-3 NIHE launched a large controlled trial of the clinical efficacy of this vaccine in approximately 100,000 persons over the age of one year in the city of Hue, a coastal Vietnamese city with endemic cholera. The analysis of this trial, performed by Branch investigators, revealed that during an outbreak of El Tor cholera 8-10 months after vaccination, a complete, two-dose regimen of the vaccine conferred 66% (95% CI: 46%, 79%) protective efficacy against hospitalized cholera; protective efficacy was similar for children aged 1-5 years (68%) and for older persons (66%). These findings lend encouragement to the notion that an inexpensive, locally produced, and effective oral cholera vaccine may be within reach of the limited health care budgets of poor countries with endemic cholera.

This earlier WC vaccine has now been augmented to provide coverage against the new 0139 serogroup of cholera, as well as the conventional 01 serogroup. Branch investigators recently evaluated a two-dose regimen of this new vaccine in a placebo-controlled, randomized trial in 143 Hanoi adults. The vaccine was found to be devoid of side effects; sera are currently being tested for vibriocidal antibody titers to enable assessment of vaccine-induced immune responses. A similar Phase II study will be conducted in Hanoi children aged 2-12 years; if this study demonstrates the vaccine to be acceptably safe, a large scale effectiveness trial of this vaccine will be launched in the spring of 1997 in approximately 200,000 persons residing in the central coastal city of Nha Trang.

6.3.3 Cholera vaccination in refugee camps

Following the 1994 epidemic of cholera among Rwandan refugees in Goma, Zaire, which resulted in over 58,000 cases and nearly 20,000 cholera deaths, and at the request of the World Health Organization, Branch investigators conducted a study to determine the cost-effectiveness of different intervention strategies to prevent and control cholera epidemics in refugee settings. Preliminary findings from this analysis indicate that in the absence of a vaccination intervention to control cholera in refugee settings, a preemptive treatment strategy, in which a treatment infrastructure and treatment supplies for managing a cholera outbreak are provided at the inception of a camp, is expected to cost less and save more lives than the currently used reactive treatment strategy, whereby necessary treatment is arranged for only when a cholera epidemic is detected. If vaccination against cholera is to be incorporated into a treatment strategy, strategies entailing preemptive vaccination (vaccination of the whole refugee camp at inception of the camp) in concert with preemptive treatment prevent the most cholera cases and the most cholera deaths, albeit at considerable cost above the preemptive treatment-only strategy. Final analyses, including sensitivity analyses, are in progress.

6.3.4 Protective relationships between _Vibrio_ _cholerae_ 01 and _Vibrio_ _cholerae_ 0139

Until 1992, it was thought that epidemic cholera was caused only by _V._ _cholerae_ serogroup 01. In 1992, however, a new serogroup of cholera, termed 0139 Bengal, caused a massive epidemic of cholera, causing tens of thousands of cases and thousands of deaths during a period of several months as it passed from countries as far west as Pakistan and as far east as China. An important question regarding _V._ _cholerae_ 0139 is whether immunity induced by earlier infection by the seventh pandemic serogroup, _V._ _cholerae_ 01, confers protection against infection by the new 0139 serogroup vibrios. The Matlab field studies area of the International Centre for Diarrhoeal Diseases Research, Bangladesh, provides an ideal setting for gathering epidemiological evidence bearing on this question, since this area is hyperendemic for cholera, including both the 01 and 0139 serogroups, and since this population has been under systematic surveillance for cholera, as well as comprehensive demographic surveillance, for over 30 years. Branch investigators are analyzing recurrent episodes of cholera documented in residents of Matlab to assess whether infection by O1 serogroup protects against later infection by the O139 serogroup, and, if so, what the duration of this protection is. The results of this study will have important implications for the potential of recently developed O1 serogroup cholera vaccines to protect against V. cholerae O139.

6.4 Helicobacter pylori

6.4.1 Helicobacter pylori in children

By the age of ten years most children in developing countries have been infected by Helicobacter pylori. Identification of clues to modes of transmission of this organism to children, as well as evaluation of the sequelae of childhood infections, thus constitute important research priorities for developing countries. Branch investigators recently completed a study that evaluated demographic, socioeconomic, and hygienic factors associated with acquisition of infection by H. pylori early in childhood among Bangladeshi children aged 2-5 years, and that assessed whether infection by H. pylori was associated with poorer nutritional parameters in these children and in an older group aged 6-9 years. The study was designed as a random, population-based survey of a rural Bangladeshi population, in which 257 children aged 2-5 years and 312 children aged 6-9 years were sampled. Using the presence of serum IgG anti-H. pylori antibodies as an indicator of H. pylori infection, the study found that 48% of children aged 2-5 years had been infected. Infected children were similar to non-infected children in this age group with respect to socioeconomic level, family access to tubewell water, and family ownership of a latrine. However, families of infected children had more persons per sleeping room in the home (3.8 vs. 3.2, $P<.05$) and were more likely to be Hindu (20% vs. 10%, $P<.05$). Infected children did not differ significantly from non-infected children in Z scores for weight-for-age (-2.66 vs. -2.78), weight-for-height (-1.17 vs. -1.28), or height-for-age (-3.58 vs. -3.56). Analysis of survey children aged 6-9 years also revealed similar nutritional indexes among infected vs. non-infected children. These data suggest that household crowding and behaviors that differ between Hindus and Muslims, but not lack of access to clean water and latrines, may enhance the transmission of H. pylori to rural Bangladeshi children. Although confirming the high frequency of infections in young Bangladeshi children, the findings do not support the notion that H. pylori is responsible for the high prevalence of malnutrition in this setting.

6.5 Enterotoxigenic Escherichia coli

6.5.1 Studies in Egypt

Enterotoxigenic Escherichia coli (ETEC) has been estimated to cause approximately 700,000 deaths of infants and children worldwide each year. Branch investigators, in collaboration with the U.S. Navy (NAMRU-3 Unit) and the University of Alexandria, have established two potential field sites in Egypt for the study of ETEC vaccines: one in Abees, near Alexandria, and one in Abu Homos, Beheira Governorate. The Abees site is periurban and relatively affluent; the Abu Homos site is rural and poor. In both sites birth cohorts are being followed biweekly to ascertain the incidence of ETEC diarrhea to enable planning of ETEC vaccine trials. In addition, in the Alexandria site the occurrence of ETEC diarrhea, classified by toxin and colonization factor virulence factors, will be related to antecedent titers of infection-induced serum antibodies to these virulence factors. These studies are providing crucial information about the incidence of ETEC diarrhea for design of future field trials of ETEC vaccines, and will also assist in the selection of measures of serological immunity for evaluating vaccine immunogenicity.

During follow-up of 242 children under three years of age in Abees from November, 1993 until September, 1995 the overall incidence of diarrhea was 2.9 episodes per child-year, ranging from 4.9 episodes per child-year in infants, to 2.5 episodes in children 12-23 months of age, and to 1.0 episode in children in the third year of life. ETEC was isolated in 125 (20%) of the 628 detected diarrheal episodes; the incidence rates of ETEC diarrhea (episodes per-child year) in the first, second, and third years of life were 1.0, 0.6, and 0.1, respectively. Most (53%) of ETEC isolated from diarrhea episodes produced only heat stable toxin (ST); 34% produced only heat-labile toxin (LT); and 13% produced both toxins. A colonization factor antigen (CFA) was detected ETEC isolates from 25% of ETEC episodes; CFA/IV was the most common CFA, accounting for 59% of CFA-positive ETEC episodes.

During follow-up of 288 children under three years of age in Abees from February, 1995 until February, 1996 the overall incidence of diarrhea was 6.0 episodes per child-year, ranging from 7.8 episodes per child-year in infants, to 5.8 episodes in children 12-23 months of age, and to 3.5 episodes in children in the third year of life. ETEC was isolated in 290 (24%) of the 1232 detected diarrheal episodes; the incidence rates of ETEC diarrhea (episodes per-child year) in the first, second, and third years of life were 1.5, 1.6, and 0.8, respectively. Most (54%) ETEC isolates from ETEC diarrheal episodes produced only heat stable toxin (ST); 30% produced only heat-labile toxin (LT); and 16% produced both toxins. A colonization factor antigen (CFA) was detected in 34% of ETEC isolates from these episodes; CFA/IV was the most common CFA, accounting for 53% of CFA-positive ETEC episodes.

These results show a strikingly higher burden of overall and ETEC-related diarrhea in the Abu Homos site than in the Abees site. Despite this difference, however, the distribution of ETEC by toxin- and CFA- phenotypes was nearly identical for both sites, as was the proportion of all diarrheal episodes attributed to ETEC. Because of the higher disease burden, the Abu Homos site has been selected for future vaccine evaluations.

In September, 1995 Branch investigators completed a study of the safety and immunogenicity of the first oral vaccine to be developed for human ETEC. This vaccine, which is killed and is given in two doses, consists of cholera toxin B subunit (which is immunologically cross-reactive with ETEC heat labile toxin B subunit), together with killed ETEC cells that express three ETEC colonization factors (CFA I,II, and IV). Designed as a randomized, placebo-controlled, clinical trial, the study enrolled 74 Egyptian adults. No severe adverse events were noted post-vaccination, and no significant differences in the proportion of subjects experiencing symptoms was noted in vaccinees vs. placebo-recipients. Over 80% of vaccinees exhibited peripheral blood IgA-antibody secreting cell responses to the B subunit, and to all component CFAs except for CS2 (69%) component of CFA II. Based on these favorable results, a similarly designed study was conducted in approximately 100 children 6-12 years of age in April, 1996. This study again revealed no side-effects that could be attributed to vaccination; analyses of immune responses to vaccination are in progress. Sequential placebo-controlled ETEC vaccine trials studies in younger children (2-5 years) and in infants are planned. The latter will be designed as a Phase

II/III trial, which will evaluate not only vaccine safety and immunogenicity, but also vaccine protection against ETEC diarrhea.

6.5.2 Studies in Bangladesh

Case-management of ETEC diarrhea with suitable rehydration therapy can avert deaths due to dehydration in children infected by ETEC. Unfortunately, many children in developing countries fail to receive adequate rehydration for diarrheal illnesses, and even appropriate rehydration will not necessarily prevent the nutritional faltering that has been associated with ETEC diarrhea. For these reasons, interventions to prevent ETEC diarrhea constitute important priorities for developing countries. Proper breast-feeding of infants and children, if effective in preventing ETEC diarrhea, would be a particularly attractive intervention, since promotion of breast-feeding has already been accorded an important role in primary health care programs in developing countries. Branch investigators assessed the relationship between breast-feeding and the risk of life-threatening (ETEC) diarrhea among Bangladeshi infants and young children under 36 months of age in a case-control study conducted in the Matlab field studies area of the International Centre for Diarrhoeal Disease Research, Bangladesh. 168 cases with clinically severe ETEC diarrhea, detected in a treatment center-based surveillance system of Matlab during 1986-86, were compared for the frequency of antecedent breast-feeding patterns with 3,679 controls selected in three random surveys of Matlab during the same calendar interval. Compared with other feeding modes, exclusive breast-feeding of infants was associated with significant protection against severe ETEC diarrhea (Relative Risk (RR)= 0.51; $P<.05$; 95%CI: 0.28, 0.96). However, during the second and third years of life, the risk of this outcome was similar in breast-fed and in non-breast-fed children (RR= 0.98;; 95%CI: 0.45,2.12), and no significant overall protective association between breast-feeding and severe ETEC diarrhea was evident during the first three years of life (RR= 0.86; 95%CI: 0.43,1.74). These findings suggest that efforts to promote breast-feeding will not exert an important preventive impact on the overall problem of life-threatening ETEC diarrhea. Other interventions, such as immunization and education about proper food hygiene, may be more logical preventive strategies for this important pediatric disease.

6.6 Typhoid fever

6.6.1 Evaluation of Vi-conjugate typhoid vaccine in Vietnam

Typhoid fever, a serious disease caused by *Salmonella typhi*, remains a major public health problem in many countries. A new typhoid vaccine developed in the Laboratory of Developmental and Molecular Immunity of NICHD, in which Vi polysaccharide of *S. typhi* is conjugated to *Pseudomonas aeroginosa* exoprotein A (Vi-rEPA), has been found to be safe and immunogenic in U.S. adult volunteers. Branch investigators are collaborating with Laboratory investigators in conducting a phase II study of the Vi-rEPA conjugate vaccine in Vietnam. A baseline epidemiological study of typhoid fever is also in progress in communities in the Mekong delta, where typhoid fever is highly prevalent, in order to establish a field site for the evaluation of vaccine efficacy. A phase III field efficacy trial is planned.

6.6.2 Typhoid epidemiology in India

Because typhoid fever is endemic in many parts of India, the Government of India has accorded high priority to the evaluation of new typhoid vaccines that could be added to the country's vaccine program. As part of the Indo-U.S. Vaccine Action Program, a research collaboration between the Government of India and the U.S. Public Health Service, Branch investigators are collaborating with two Indian institutions, the All India Institute of Medical Sciences in Delhi and the Tuberculosis Research Center in Madras, on the development of field sites for the future testing of typhoid vaccines. The site in Delhi is urban, while that near Madras (Chingleput) is rural. In the first phase of this project, now in progress, a population of about 19,000 persons has been demarcated in Delhi, of which 8,000, in 20 equal-sized clusters, is under combined active (twice-weekly home visits) and passive surveillance for typhoid fever, and 11,000 is under passive surveillance only. In Chingleput, 9,000 persons, from 8 villages, are under combined active-passive surveillance, while 11,000 persons, from 12 villages, are under passive surveillance only. During the initial 6 months of surveillance in Delhi, the overall incidence of blood culture-confirmed typhoid fever, per person-year of follow-up, was 2.9 per 1,000; however, the incidence among subjects under combined active-passive surveillance was 4.6 per 1,000 while that for subjects under only passive surveillance was only 1.6 per 1,000. Interestingly, for the population under combined surveillance, 11 of 19 (58%) cases were aged five years or under, while the corresponding figure for the population under only passive surveillance was 1 out of 9 (11%) cases. These data from Delhi indicate an extremely high overall incidence of blood culture-confirmed typhoid; a clear augmentation of the detection of typhoid by adding active surveillance procedures; and a substantial incidence of typhoid in the under-five population-- a fact missed when active surveillance is not undertaken. Surveillance has only recently begun in Chingleput, and it is too early to draw any conclusions about the occurrence of typhoid fever in this site. Additional substudies are being undertaken in each site to assess, using case-control studies, the relative importance of foodborne vs. waterborne transmission of *S. typhi*; and to evaluate the long-term disability and economic consequences of typhoid fever in the two populations. It is already clear that the incidence of typhoid in Delhi is adequate to conduct efficacy trials of typhoid vaccines, and such trials are now being planned for the next phase of the project.

6.7 Vaccination of U.S. infants

6.7.1 Age-appropriate immunization in the District of Columbia

Well child care is the primary means by which preventive health care is provided to infants and young children in the United States. National recommendations for well child visits correspond with the recommended child vaccination schedule. Unfortunately, about half of infants and young children in U.S. cities fail to obtain the appropriate immunizations, partly due to low rates of participation in well child care. Barriers to attending well child care are known to exist among low-income populations. However, well child care and immunizations are commonly provided free of charge to low-income patients. The purpose of this study is to identify psychological, sociological and other factors associated with low rates of immunization and well child care among low income families in the District of Columbia, in order to assist in the formulation of future interventions that can be applied to low income, urban populations. The study is prospectively evaluating determinants both of the initiation of immunizations at two months of age and of continuation of age-appropriate immunizations through seven months of age in a cohort of 480 mother-infant dyads. The prospective study design will allow assessment of predictors ascertainable shortly after birth, the time most relevant for the initiation of interventions. Field work for this study began in the Fall of 1995 and will continue through the Spring of 1997.

6.7.2 Conventional vaccination of HIV-infected infants

Although some evidence points to reduced responses to conventional infant vaccines in HIV-infected infants, this evidence is based on small studies. Branch investigators are analyzing sera obtained during the Women-Infant HIV Transmission Study (WITS) to assess responses to primary series of diphtheria-tetanus-pertussis (DTP) and PRP-conjugate vaccines against Haemophilus influenzae type b (Hib), administered early in infancy, in HIV-infected versus non-infected children. In the WITS study, infants are recruited if they are born to mothers documented to be HIV-infected. These infants are then followed longitudinally and bled at systematic intervals. The present analysis is assessing serum antibodies to diphtheria and tetanus toxoid, Hib capsular polysaccharide (PRP), and pertussis agglutinins in bloods drawn 2-4 months after primary series of DTP and PRP-conjugate vaccines, and again about one year later. Because of the design of the WITS study, these analyses permit comparisons of HIV-infected infants versus non-infected infants who are well-matched for important biological and sociodemographic variables. Preliminary analyses have revealed significantly lower titers of serum IgG anti-PRP antibodies, which are the mediators both natural and vaccine-induced protection against Hib. Further analyses are now exploring whether these differences represent poorer performance of particular Hib vaccines in infected infants, or whether the differences can be attributed to less comprehensive vaccination of these infants.

6.7.3 Pneumococcal vaccination of HIV-infected infants

Newborns infected by HIV are expected to have up to a 25% risk of at least one invasive pneumococcal infection before reaching the age of two years. However, conventional pneumococcal polysaccharide vaccines, which are T cell independent antigens, are not suitable interventions to prevent these serious infections, since children typically do not mount adequate serum antibody responses to the serotypes contained in the vaccine until they reach 18-24 months of age. Another approach would be to conjugate pneumococcal polysaccharides to protein carriers, to convert the polysaccharides to T cell-dependent antigens, which should be capable of eliciting ample antibody responses even when given during infancy. Branch investigators are currently collaborating in a multicenter randomized, placebo-controlled trial in HIV-infected infants to evaluate a new pneumococcal vaccine, which contains the polysaccharides of seven of the most prevalent pneumococcal serotypes, conjugated to a mutant diphtheria toxoid protein. Three doses are being administered at two-month intervals, and both the safety of the vaccine and serotype-specific serum antibody responses are being assessed. In addition, the study is evaluating whether vaccination reduces the risk of nasopharyngeal colonization by pneumococcal serotypes contained in the vaccine.

6.8 New methods for vaccine evaluations

6.8.1 New statistical models for vaccine trials

Branch investigators have completed a collaborative study with investigators at Emory University on a project to evaluate the use of new statistical models for analyzing vaccine trial data. In this analysis, the models were employed to distinguish between two different types of temporal trends of declining vaccine protection: continuous waning of protective efficacy versus piece-wise waning. Using data collected in the Bangladesh trial of killed oral cholera vaccines, the analysis revealed that for the killed whole cell-only (WC) vaccine, the former was the appropriate model, whereas for the B subunit-killed whole cell (BS-WC) vaccine the latter was most appropriate. These analyses, which illustrate the utility of these models for analysis of vaccine trial data, are concordant with the biological observation that the B subunit component of the BS-WC vaccine conferred initial short-lasting incremental protection above that attributable to the killed whole cells *per se*.

6.8.2 New criteria for case-control studies of vaccine effectiveness

Case-control studies are increasingly relied upon to provide evidence about vaccine effectiveness after licensure, and recently have also been used in applications for licensure of new vaccines that cannot be ethically tested in randomized, controlled trials. However, in contrast to randomized, controlled trials, for which there are well-defined criteria for judging the methodological adequacy of studies, no such criteria exist for case-control studies of vaccines. Branch investigators have recently developed a set of such criteria, and are now applying them to published case-control studies in order to assess aspects that require particular attention in future studies.

6.8.3 Establishment of methods for vaccine effectiveness trials

Despite the profusion of promising new vaccines against illnesses prevalent in developing countries, uncertainties about the balance between costs and benefits of new vaccines have retarded their use in public health practice. Conventional prelicensure trials of vaccine protection exacerbate these uncertainties by focusing on measurement of vaccine efficacy, or the performance of a vaccine under idealized conditions. In a recent publication in *The Journal of the American Medical Association*, Branch investigators outlined how innovatively designed vaccine effectiveness trials can provide a more pragmatic perspective by addressing the performance of vaccine under the ordinary conditions of a public health program, by capturing direct as well as indirect effects of vaccination, and by comprehensively addressing outcomes of public health concern. The use of effectiveness trials should enable more rational triaging of new vaccines for developing countries, and may accelerate the introduction of new vaccines into public health practice by preventing speculative debates about practical costs and benefits.

6.9 Ear infections and respiratory conditions

Branch staff have collaborated with the National Institute on Deafness and Communicative Disorders to evaluate factors affecting the risk of frequent ear infections, hearing impairment, and deafness in U.S. children. The results of this study indicate that otitis media is more prevalent in males than in females.

The rates are more than twice as high among whites than among blacks, possibly due to differences in access to medical care. Hearing impairment and deafness is more prevalent among children with low birth weight and is reported less frequently in those without health insurance or not on Medicaid. These findings are in press in the Proceedings of the Sixth International Symposium on Recent Advances in Otitis Media. In another study, Branch investigators are collaborating in an evaluation of serum cotinine levels obtained from the National Health and Nutrition Examination Survey. The study will examine the association of environmental tobacco smoke with ear infections and respiratory conditions in U.S. children under age six.

Prevention Research Branch

The Prevention Research Branch (PRB) was formed in 1990 to foster the development of a program of research in health promotion and disease prevention in maternal, child and adolescent health. The Branch evolved as a new component of the Division without preexisting staff (the Branch Chief joined the Institute in April 1990 to establish this effort and two senior staff were appointed in August 1992).

Programmatically, the Branch directs its efforts to the initiation, design and conduct of biobehavioral research testing the efficacy or effectiveness of behavioral, educational and environmental strategies and communications processes to effect child and adolescent health, including surveys, randomized trials and demonstration education programs. The PRB develops original biobehavioral research studies with the dual objectives of testing and applying behavioral change theory for the improvement of individual and community health. The projects are conducted using a variety of mechanisms, including the contract mechanism, with development of the detailed research protocols, project implementation, data analysis and preparation of manuscripts undertaken collaboratively between Branch investigators and the contractor. The primary focus of the PRB research is the: a) development and evaluation of interventions for maternal and child health conducted in primary health care settings, including enhancement of the capacity of primary care physicians to counsel parents in prevention and health promotion for children; and b) testing of school based-programs for prevention of problem behavior and health promotion in children and adolescents.

Staff Changes

Currently the Branch has two Senior Staff. There are four Intramural Research Training Award (IRTA) Postdoctoral Fellows.

Health Promotion and Disease Prevention in Primary Health Care Settings

Prevention of Childhood Injury Among Preschool Children

Unintentional injuries from burns, drownings, pedestrian injuries and ingestions are the leading cause of death among U.S. children ages 1 to 4 years, especially among disadvantaged children. Recent data show that almost 25% of children received medical care for nonfatal injury from time of birth to 3 years of age. In children 1 to 4 years, 31.7% have medically attended injuries annually (one third of which are severe and require surgery, bed restriction and loss of normal activity).

Prevention of unintentional childhood injures through heightening the awareness of parents of associated risk factors and increasing counseling by primary health care providers on safety and injury prevention is a national health priority. Injury prevention counseling in the primary care setting can contribute to childhood injury prevention and is recommended by the American Academy of Pediatrics (AAP) to be included as part of routine health supervision.

Studies consistently demonstrate the influential role of primary care physicians in their counseling and practice behaviors on the adoption of recommended practices by patients, including health protective behaviors undertaken by parents for children. In a recent national survey, 60% of parents report never receiving any injury prevention counseling from their child's primary care physician. Prior research of PRB investigators suggests that: (a) education of physicians can successfully lead to changes in their practice behaviors and the behavior and health status of their patients; and (b) psychosocial factors which may be amendable through intervention are associated with childhood injury.

PRB investigators are designing a randomized trial to determine if a multi component educational intervention can modify and increase the injury prevention/health promotion counseling and advocacy practices of nonspecialist pediatricians and reduce unsafe practices for childhood injuries in the home by parents, subsequently reducing unintentional injury morbidity in children. The primary analysis of interest will be to assess the effect of the provider intervention on: (1) improvements in physicians' performance as motivators and educators for injury prevention/health promotion; 2) decreased morbidity in their pediatric patients (i.e., medically attended nonfatal injuries); 3) reduction in unsafe practices in the home associated with childhood injuries by parents; and 4) increased health protective behaviors by parents for their children.

An experimental design will be employed with physicians assigned to no treatment (control) or two levels of an injury prevention education intervention. A parent-child sample will be obtained for each pediatrician. The study will focus on children 0 to 3 years of age.

MD/Family Partnership: Education in Asthma Self-Management

A strong partnership between physicians and patients has been discussed as a crucial part of asthma self-management. Both under-treatment and lack of effective patient education by physicians are cited as reasons that asthma related problems are on the increase. PRB investigators are collaborating with investigators at the University of Michigan School of Public Health and Columbia University School of Medicine in a study to develop and test an intervention based on theories of self-regulation of behavior in changing the diagnosis and treatment patterns and educational behavior of physicians who treat children with asthma.

It is hypothesized that education for physicians, based on the principle of self-regulation provided through an interactive seminar, will be effective in changing physicians' clinical behavior and improving their performance as health educators and counselors. The study also assesses the effects of education for pediatricians on the self-management behavior, health status, and health care utilization of their patients.

A randomized experimental design involving 60 pediatricians and 650 parent-child pairs from their practices is being employed. Data is being collected in Ann Arbor, Michigan and New York City. The study will be conducted over a 5 year period. Baseline information has been collected from 60 physicians and 637 parent-child pairs, 28 physicians attended the educational sessions, and 3 months post-intervention follow-up data has been collected from health care utilization sources, parents and physicians. Baseline and follow-up data are currently being analyzed. The 12 months post-intervention follow-up will be completed in January 1997. PRB researchers are undertaking an analysis of pediatrician self-monitoring for patient communication practices and efficacy perceptions as correlates of their counseling and education communication behaviors.

Parent-Child Interaction and Asthma Self-Management

Although effective medical self-management strategies are available for control of asthma in children, pediatric asthma morbidity and mortality has increased. The existing approaches for enhancing patient responsibility in chronic disease management (patient education; altering treatment regimens; behavioral techniques) do not address the developmental needs of pediatric patients. Currently little information is available on the process of parent-to-child transfer of responsibility for asthma self-management. This study employs concepts from the theory of L. Vygotsky of shaping child cognitive development in the "zone of proximal development" (joint activity between children and the "experts") and from Bandura's social learning theory for shaping child behavioral development. Understanding the process of parent-to-child transfer of asthma self-management behavior will provide suggestions for the development of effective interventions for pediatric asthma self-management programs.

The overall goal of this study is to determine the components of parent-to-child transfer of asthma self-management behaviors (parental definitions of child adherence, attitudes toward transferring responsibility and strategies employed) and whether the adoption of self-management behaviors by young adolescents (ages 8-13) with asthma influences their health status and health care utilization. The specific aims are to examine: 1) distribution of responsibilities (self-management activities performed by the child, by the mother, or jointly); 2) factors leading to the current distribution of activities; 3) joint decision-making for asthma-related issues; and 4) maternal approaches to teaching asthma self-management skills. The study is a separate component to the MD/Family Partnership: Education for Asthma Management study. It is being conducted in collaboration with Noreen M. Clark, Ph.D., School of Public Health, the University of Michigan.

The study is divided into two phases. Phase I is qualitative and involves individual, exploratory face-to-face interviews with a subsample (N=32) of mothers and their children (ages 8-13) with asthma. In Phase II, the information gathered through the focus interviews will be used to develop questions relevant to the study for telephone interviews with a larger sample of mothers and children (N=293 parent-child pairs) from the project. Phase I data collection was completed in June 1996 and is being analyzed. Phase II data collection is scheduled for November 1996 through March 1997.

In the exploratory interviews, mothers describe barriers to their child's adherence with self-management practices (e.g., denial of asthma, lack of perceived vulnerability to the consequences of not undertaking self-management behaviors, dependence on parents for management) and lack of correct self-management practices by the child (e.g., ignoring asthma symptoms, failing to take medication, exposure to asthma triggers). Most parents recognized the importance and benefits of transferring responsibility for self-management and many gave examples of successful transfer. Some parents were reluctant to transfer self-management responsibility because of concerns about the child implementing the procedures or beliefs that parents were responsible for assuring adherence. In response to questions assessing strategies parents use to enhance the capacity of their asthmatic children to self-management, successful strategies were those that involved the child in self-management as a joint activity, provided support and organization for the child and the use of motivators (e.g., positive reinforcement, child's desire for autonomy).

School-Based Health Promotion and Disease Prevention Programs for Children and Adolescents

The school setting provides an attractive context for health behavior change interventions. The mandatory presence of students in schools offers an excellent opportunity for longitudinal research and treatment programs can be integrated into the general educational curriculum with potential influence on large numbers of individuals with modest investment of professional resources. Maintenance of behavior change may be enhanced to the extent that school-based programs enable greater environmental support to foster and sustain behavior change.

Preventing Problem Behavior Among Middle School Students

The dramatic increase in the prevalence of problem behaviors that occur during adolescence is a national concern. Early adolescent problem behavior, including school misconduct, failure, and dropout; tobacco, alcohol, and other drug use; violence, delinquency, and other antisocial acts, places youth at an increased risk for school failure, involvement in the criminal justice system, and chronic substance abuse. While a small proportion of conduct disordered youth are responsible for a disproportionate amount of problem behavior, the majority of adolescents report engaging at least occasionally in problem behaviors, such as alcohol use, vandalism, and physical fighting. This population of experimental or occasional problem behaving youths may benefit from preventive interventions in which they are able to improve behavioral skills and social competence and develop supportive social environments to resist antisocial pressures and obtain reinforcement for prosocial behavior, thereby, improving their chances for a productive and healthful adolescent experience.

In collaboration with Behavior Health Research, Inc. (BHR), the contractor, and Charles County School District, PRB investigators are conducting a study to implement and test the efficacy of a multi-component program of school-based interventions in preventing problem behavior in a sample of middle school students. The study is unique in its focus on multiple problem behaviors and multiple interventions conducted over a 3-year period. The primary research question is "What is the effect on the frequency of problem behavior among students exposed to the special intervention that includes nontraditional curriculum, school environment and parent education compared to students in the comparison group?" A secondary question is "What psychosocial and environmental factors are associated with problem behavior?" Specific aims of the study are to evaluate: (a) the extent and quality of implementation of the intervention components; (b) the impact of the intervention components on selected mediating variables, including student and parent self-efficacy, expectancies, and perceived norms; and ⓒ the impact of the intervention on the extent of student problem behavior.

A randomized quasi-experimental design is being used to test the problem behavior protective effects of exposure to a social skills curriculum, enhanced school environment, and parent education in the special intervention group compared to problem behavior in a comparison group receiving usual education only. Approximately 3000 6th-7th-8th graders in 2 cohorts in 7 middle schools will be involved in the study, approximately half of whom will receive the special intervention.

The primary outcomes of the study are the extent of problem behavior, including alcohol, tobacco, and drug use, sex, violence, school misbehavior, truancy, vandalism, and crime. Proximal measures of the success of the intervention include prosocial behavior, school bonding, family and peer relations, social adjustment, and parenting behavior, as well as the extent and quality of implementation.

The study is currently collecting baseline data prior to delivery of the 6th grade intervention. One middle school (6th grade) served as the pilot test site for needs assessments, student and parent measures, and the curriculum, school environment, and parent education intervention components. Baseline data collection is scheduled for September, 1996.

Preliminary descriptive data from the pilot tests show that only one or two students reported using tobacco, alcohol or marijuana, whereas stealing, fighting, bullying, graffiti, and going someplace dangerous were common. A large number of students intended to smoke, drink, or go dangerous places sometime during middle school and many intended to do so in high school. Many students perceived that most high school students smoke and drink alcohol as well as that at least one of their five closest friends cheated in school or bullied other kids (a few reported that their friends stole, defaced property, or lied to their parents about where they were or who they were with). When asked about school climate, most students agreed that their teachers were fair and school rules were clear, but many reported that students did not respect each other, that the building was not pleasant, and school topics were not interesting. Less than half indicated that they enjoyed class work and barely half indicated they were happy at school.

Collaborations with the NIH-DC Initiative to Reduce Infant Mortality

Pregnancy Prevention Among Minority Adolescents

As a part of the NIH-DC Initiative to Reduce Infant Mortality, PRB investigators are participating in a study to test the efficacy of a program of intensive interventions to prevent unwanted pregnancies among African American and Hispanic adolescents. Other target outcomes include the delay of initiation of intercourse, prevention of STDs, use of condoms and other contraceptives, and increased use of general medical and reproductive health services.

In conjunction with this effort, formative research involving focus groups with African American female and male adolescents was conducted to better understand the cognitive and social factors that impact upon sexual and contraceptive behavior. Five focus groups were conducted with nulliparous, female clinic attenders (n=37) and six focus groups were conducted with males recruited from a youth employment program (n=54) in order to develop hypotheses regarding barriers to behavior modification.

The adolescent females were 14 to 17 years and predominantly in school (93%). Seventy-nine percent had ever had sexual intercourse, and the median age of initiation was 14. Fifty-nine percent of sexually active respondents had more than one partner in the past year. Most sexually active respondents had ever used a birth control method, and condoms were the method most frequently reported (70%).

For female participants, social norms and social influences impacting upon sexual behavior were manifest through verbal pressure from males and females to engage in sexual intercourse. Such influence is strengthened by perceptions of sexual involvement as normative. Similar to the literature suggesting that involuntary sex is often reported among young sexually active teenagers, study females reported involuntary sex as a problem, particularly for females who are not empowered to assert themselves in interactions with males.

Exploration of pregnancy-related beliefs and their relationship to social influences showed that although pregnancy is viewed as normative, the adolescent female participants recognized the impact of pregnancy for a teenager with ambitious life goals. Although participants stated that some of their peers wanted to become pregnant, expecting that a baby would bring them status, love from the child, or attachment to a male, the data suggest that often teens do not desire pregnancy.

Male focus group participants were older (16-21 years), more likely to be sexually active (98%), and earlier initiators of sexual activity (median age 13) than female participants. Seventy-one percent of males had two or more partners in the past year and 42% reported having ever gotten a girl pregnant.

Male focus group data suggest that peer influence imparted the need to project the image of sexual involvement. It was acceptable to have both "main" and "side" partners, and condoms were utilized 44% of time with the former and 48% of the time with the latter. The adolescent males described the availability of sexual partners, and reported that most of their peers are not interested in procreation but that typically pregnancies result from lack of attention to the potential consequences of sex.

Immunization Among DC Infants

Another NIH-DC Initiative to Reduce Infant Mortality project involving PRB investigators is a study entitled "Lack of Non-Immunization Among DC Residents". The purpose of this research is to identify the determinants of lack of age-appropriate immunizations. The subjects are low-income mothers of minority infants born in the District of Columbia. A prospective cohort study is underway, involving 480 subjects in three DC hospitals (DC General Hospital, Howard University Hospital, and Washington Hospital Center). Patients are interviewed in the hospital after giving birth and again at 3-months and 7-months postpartum. Medical record information on the mother and baby will be abstracted. Data collection is scheduled for completion in August 1997.

Other Studies

Survey of Parental Behaviors to Prevent Adolescent Alcohol Use

Underage drinking and drinking and driving pose significant risks to adolescent health and well being. Parental monitoring is one of many factors that are important for understanding and addressing this significant health problem. The purpose of this study is to examine the associations between parental monitoring and adolescent alcohol consumption, drinking and driving, and riding with an intoxicated driver. Questions of particular interest include: 1) the relationship between parent monitoring and enforcement of rules regarding alcohol use and adolescent's self reports of use; 2) parents awareness of their adolescents drinking in relationship to their monitoring behaviors; 3) concordance between parent and adolescent reports of parents' monitoring practices as related to adolescent behavior; and 4) age differences in parenting practices regarding adolescent drinking. Understanding current parenting practices in relation to adolescent behavior is an important step in designing effective interventions to address underage drinking and adolescent drinking and driving.

Telephone interviews were conducted with random sample of 455 parents and their adolescent children (ages 14-19) in Maryland. Parents were asked about their teen's behavior and their strategies for parenting. Adolescents were asked to report on their behavior and their perceptions of their parents' reactions to their misbehavior. The study is being conducted in collaboration with Kenneth Beck, Ph.D., Department of Health Education, University of Maryland.

The majority of adolescents (58%) reported drinking in the last 12 months, and the rates of self-reported drinking increased with age (40% of 14 year olds and 75% of 19 year olds). During the past 12 months, 25% of adolescents reported having ridden with a drinking driver and 6% of those teens with drivers licenses reported drinking and driving. Parents perceived themselves as vigilant in monitoring their children's behavior, with most reporting that they knew when, how and with whom their child spent free time, imposed a curfew and knew when their child came home. Teens also perceived their parents as doing a high level of monitoring. Both parent and teen reports of parent monitoring decreased as teen's age increased with significantly less monitoring reported for 18 year olds than for all other age groups (parent report: $F = 18.95$, $p<.0001$; teen report: $F = 15.86$, $p<.0001$). Nevertheless, parents were unaware of their adolescents' underage drinking (60% of adolescents reporting drinking had parents who reported that their child never drank) and parental lack of awareness was most pronounced for those adolescents in the youngest ages.

Perceptions of Parents Regarding the Onset of Sexual Intercourse Among Youth

Twenty-three percent of US adolescents have initiated sexual intercourse by age 14. It has become more popular to restrict the provision of sensitive reproductive health information and services to adolescents in deference to parents' rights to make judgments about what is appropriate for their children. However, little knowledge is available regarding the role that parents play in providing sexuality education for early adolescents and the barriers parents face in doing so.

The purpose of this study is to explore views about youth sexuality, sexuality education, and pregnancy/STD prevention from the perspective of mothers of high-risk early adolescent females, and to examine mother-daughter communication around sexuality to characterize mothers' predominant messages, sense of efficacy and comfort, and extent of involvement. The study is being conducted collaboratively with the Center for Adolescent Health Promotion, Johns Hopkins University under the direction of Dr. Cheryl Alexander.

African American mothers or female caretakers (n=30) of 13-14 year old girls were recruited through an urban school and a community service organization to participate in one-on-one in-depth interviews.

Eighty percent of participants were biological mothers of an early adolescent female (other caretakers were female relatives). They were predominantly single mothers of low to moderate income and 53% of the mothers interviewed had their first child before the age of 18. Fifty percent of participants completed high school or a GED program and most were not working full-time.

Preliminary findings show a high level of awareness of early teenage sexuality. Consistent with other estimates for this population, mothers estimated that 57% of girls 14 years of age or younger had ever had sexual intercourse. However, 73% of participants state that their daughter is definitely (63%) or probably (10%) not having sex. While mothers acknowledge that early sexual activity occurs, it is also clearly defined as unacceptable and problematic (i.e., mothers placed a high value on avoidance of early parenting and STDs).

Mothers almost unanimously report talking to their daughters about sex and they define this as primarily their responsibility. Although frequency and extent of discussion varies widely, mothers generally report being relatively comfortable providing general sexuality and STD information. For some mothers, a barrier to communication is their daughters' reactions to such information and guidance.

DIVISION OF EPIDEMIOLOGY, STATISTICS AND PREVENTION RESEARCH

Office of the Director

Fikree FF, Berendes HW, Villar J. A rapid nutritional evaluation of pregnant women in low socioeconomic settlements of Karachi, Pakistan. J Pakistan Med Assoc, in press.

Biometry and Mathematical Statistics Branch

Blair RC, Troendle JF, Beck RW. Control of family wise errors in multiple endpoint assessments via stepwise permutation tests. Stat Med 1996;15:1107-21.

Chang AS, Dillingham TR, Yu KF. Statistical methods of computing reference values for the side-to-side differences in nerve conduction studies. A J Phys Med and Rehab, in press.

DerSimonian R. Meta-analysis in the design and monitoring of clinical trials. Stat Med 1996;15:1237-48.

DerSimonian R, Clemens J. Does vasectomy cause cancer? Chance 1996, in press.

Kimonis VE, Troendle J, Rose SR, Yang ML, Markello TC, Gahl WA. Effects of early cysteamine therapy on thyroid function and growth in nephropathic cystinosis. J Clin Endocrin and Metabol 1995;80:3257-61.

Levine R, DerSimonian R. Effect of calcium supplementation on pregnancy-induced hypertension and preeclampsia: a meta-analysis. [Letter to the Editor]. JAMA 1996, in press.

Mukerjee R, Gupta S. Geometric construction of balanced block designs with nested rows and columns. Discrete Mathematics 1991;91:105-8.

Ng TH, Yu KF. A new decision rule in active control equivalence studies. Proceedings of the American Statistical Association, Biopharmaceutical Section, 1995:265-70.

Park CG, Park T, Shin DW. A simple method for generating correlated binary variates. American Statistician 1996, in press.

Park T. Multivariate regression models for discrete and continuous repreated measurements. Com Stat Theory Meth 1994;23:1547-64.

Park T, Brown MB. Log-linear models for a binary response with non-ignorable non-response. Computational Statistics and Data Analysis, in press.

Park T, Lee SY. A test of missing completely at random for longitudinal data with missing observtions. Stat Med, in press.

Park T, Shin MW. A practical extension of generalized estimating equations approach for longitudinal data. Communications in Statistics-Theory and Method 1995;23:1547-64.

Read JS, Troendle JF, Klebanoff MA. Infectious disease mortality among U.S. infants. Am J Public Health, in press.

Shin DY, Park CG, Park T. Testing for ordered group effects with repeated measurements. Biometrika, in press.

Strickler H, Hoover DR, DerSimonian R. Problems in interpreting HIV sentinal seroprevalence studies. Ann Epidemiol 1995;5:447-54.

Troendle JF. A permutational step-up method of testing multiple outcomes. Biometrics 1996;52:125-38.

Troendle JF, Yu KF, Herman AA. A scale conversion problem to avoid. Am J Public Health, in press.

Westhaff C, Gentile G, Lee YJ, Zacur H, Helbig D. Predictors of ovarian steroid secretion in reproductive age women. Am J Epidemiol, in press.

Yu KF. A simple comparison of two sequences of probabilities. J Stat Plan Inference 1996;52:271-87.

Yu KF. On estimating a common odds ratio under binary cluster sampling. [Letter to the Editor]. Stat Med, in press.

Computer Sciences Bibliography

Bijur PE, Trumble AC, Harel Y, Overpeck MD, Jones DH, Scheidt PC. Sports and recreation injuries in U.S. children and youth. Arch Pediatr Adolesc Med 1995;149:1009-16.

Phillips D, Huston A and The NICHD Early Child Care Network. Poverty and patterns of child care. In: Brooks-Gunn J, Duncan G, eds. Consequences of growing up poor. New York: Russell-Sage, in press.

Scheidt PC, Harel Y, Trumble AC, Jones DH, Overpeck MD, Bijur PE. Nonfatal injuries among U.S. children. [Letter to the Editor]. Am J Public Health 1996;86:892.

Vandell D and The NICHD Early Child Care Network. Characteristics of infant child care: Factors contributing to positive caregiving. Early Childhood Research Quarterly, in press.

Epidemiology Branch Bibliography

Clemens J, Albert MJ, Rao M, Huda S, Qadri F, van Loon F, Pradhan B, Naficy A, Banik A. Sociodemographic, Hygienic and Nutritional Correlates of Helicobacter Pylori Infection of Young Bangladeshi Children. Pediatr Infect Dis J, in press.

Clemens JD, Brenner R, Rao M, Lowe C. Evaluating new vaccines for developing countries: efficacy or effectiveness? JAMA 1996;275:390-7.

Clemens JD, Rao MR, Sack D, Ahmed F, Khan MR, Chakraborty J, Kay B, Huda S, Yunus M, van Loon F, Svennerholm A-M, Holmgren J. Impaired immune response to natural infection as a correlate of vaccine failure in a field trial of killed oral cholera vaccines. Am J Epidemiol 1995;142:759-64.

Collins JW, Herman AA, David RJ. Prevalence of very low birthweight in relation to income-incongruity among African-American and white parents in Chicago. Am J Public Health, in press.

Darwish M, Farris R, Clemens J, Rao M, Edelman R. Prevalence and correlates of hepatitis C infection in a Nile delta village. Am J of Trop Med Hyg, in press.

Goldenberg RL, Dubard MB, Cliver SP, Nelson KG, Blankson K, Herman AA. Pregnancy outcome and intelligence at age five. Am J Obstet Gynecol, in press.

Goldenberg RL, Klebanoff MA, Nugent RP, Krohn MA, Hillier SL, Andrews WW. Bacterial colonization of the vagina during pregnancy in four ethnic groups. Am J Obstet Gynecol 1996;174:1618-21.

Herman AA. A conceptualization of race in epidemiologic research. Ethnicity Dis 1996;6:7-20.

Herman AA, Berendes HW, Yu KF, Cooper LC, Overpeck MD, Rhoads G, Maxwell J, Kinney B, Koslowe PA, Coates D. The evaluation of a community-based enriched model prenatal care project in the District of Columbia. Health Serv Res, in press.

Herman AA, McCarthy B, Bakewell J, Ward R, Mueller BA, Maconochie N, Read A, Zadka P, Skjaevveu R. Data linkage methods used in maternally-linked birth and infant death surveillance data sets from the United States (Georgia, Missouri, Utah & Washington State). Israel, Norway, Scotland and western Australia. Paediatr Perinat Epidemiol, in press.

Herman AA, Yu KF. Adolescent age at first pregnancy and subsequent obesity. Paediatr Perinat Epidemiol, in press.

Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, Edelman R, Cotch MF, Pastorek J, Rao AV, McNellis D, Regan JA, Carey JC, Klebanoff MA. The association between bacterial vaginosis, bacteroides, and Mycoplasma hominis with preterm, low-birth-weight delivery. N Engl J Med 1995;333:1737-42.

Hoffman HJ, Overpeck MD, Hildesheim ME. Factors in the U.S. affecting risk of frequent ear infections, deafness or trouble hearing and related conditions. Proceedings of the Sixth International Symposium on Recent Advances in Otitis Media, Fort Lauderdale, FL, June 4-8, 1995, in press.

Kenney LB, Nicholson HS, Brasseux C, Mills JL, Robison LL, Zeltzer LK, Meadows AT, Reaman GH, Byrne J. Birth defects in offspring of adult survivors of childhood acute lymphoblastic leukimia. Cancer, in press.

Klebanoff MA, Clemens JD, Read JS. Maternal smoking during pregnancy and childhood cancer. Am J Epidemiol, in press.

Klebanoff MA, Read J, Mills JL, Shiono PH. Neonatal vitamin K administration and cancer during childhood. In: Sutor A, Hathaway W, eds. Proceedings of the International Symposium on Vitamin K in Infancy. Stuttgart, Germany: 1995, in press.

Krulewitch CJ, Herman AA, Yu KF, Johnson YR. Does changing paternity contribute to the risk of intrauterine growth retardation. Paediatr Perinat Epidemiol, in press.

Levine R, Esterlitz J, Raymond E, DerSimonian R, Hauth J, Curet B, Sibai B, Catalano P, Morris C, Clemens J, Ewell M, Friedman S, Goldenberg R, Jacobson S, Joffe G, Klebanoff M, Petrulis A, Rigau-Perez J: The trial of calcium for preeclampsia prevention (CPEP): rationale, design, and methods. Controlled Clin Trials, in press.

Mills JL. Birth Defects. Congressional Record 1996;141:E1743-4.

Mills JL, Scott JM, Kirke PN, McPartlin JM, Conley MR, Weir DG, Molloy AM, Lee YJ. Homocysteine and neural tube defects. J Nutr 1996;126:756S-60S.

Nagourney BA, Kramer MS, Klebanoff MA, Usher RH. Recurrent respiratory distress syndrome in successive preterm pregnancies: are some women "RDS-prone?" J Pediatr, in press.

Regan JA, Klebanoff MA, Nugent RP, Blackwelder WC, Eschenbach DA, Lou Y, et al. Colonization with group B streptococci in pregnancy and adverse outcome. Am J Obstet Gynecol 1996;174:1354-60.

Shu XO, Clemens J, Zheng W, Ying DM, Ji BT, Jin F. Infant breast feeding and the risk of childhood lymphoma and leukemia. Internatl J Epidemiol 1995;24:27-32.

Simpson JL, Mills JL, Kim H, Holmes LB, Lee J, Metzger B, Knopp R, Jovanovic-Peterson L, Aarons J, Conley M. Infectious Processes: An infrequent cause of first trimester spontaneous abortions. Human Reprod 1996;11:668-72.

van Loon FPL, Clemens J, Chakraborty J, Rao M, Kay B, Sack D, Yunus M, Khan M, Ali M, Svennerholm A-M, Holmgren J. Field trial of inactivated oral cholera vaccines in Bangladesh: results from five years of follow-up. Vaccine 1996;14:162-6.

Wassif N, Loza S, Rao M, Terreri N, Clemens J. Impact of a water-sanitation program in remote villages of Upper Egypt: Results of a large-scale community experiment. Abstract. US-Japan Cooperative Science Meeting. Kiawah, October, 1995.

Whitehead AS, Gallagher P, Mills JL, Kirke PN, Burke H, Molloy AM, Weir DG, Shields DC, Scott JM. A genetic defect in 5,10 methylenetetrahydrofolate reductase in neural tube defects. Q J Med 1995;88:763-6.

DIVISION OF EPIDEMIOLOGY, STATISTICS AND PREVENTION RESEARCH

Prevention Research Branch

Clark NM, Nothwehr F, Gong M, Maiman LA, Hurwitz ME, Evans D, Roloff D, Melins RB. Physician-patient partnership in managing chronic illness. Acad Med 1995;70:957-9.

Maiman LA, Rodewald LE. Compliance with pediatric health care recommendations. In: Hoekelman RA, Freedman SB, Nelson NM, Seidel HM, eds. Primary Pediatric Care. New York: John Wiley and Son, 3rd ed., in press.

Radziszewska B, Richardson JL, Dent CW, Flay BR. Parenting Style and adolescent depressive symptoms, smoking, and academic achievement; ethnic, SES, and gender differences. J Behav Med 1996;19:3; 289-305.

Rogoff B, Radziszewska B, Masiello T. Analysis of developmental processes in sociocultural activity. In: Martin L, Nelson K, Tobach E, eds. Sociocultural Psychology: Theory and Practice of Doing and Knowing. Crambridge,UK: Cambridge University Press, 1995;125-49.

Santelli JS, Celantano DD, Rozsenich CR, Crump AD, Davis MV, Polascek M, Augustyn M, Rolf J, McAlister AL, Burwell L. Interim outcomes for a community-based program to prevent perinatal HIV transmission. AIDS Educ Prev 1995;7:3;210-20.

Simons-Morton B, Crump, AD. Empowerment: The Process and the Outcome. Health Educ Q, 1996;23:3;290-2.

Simons-Morton, BG, Crump, AD, Donahue L. Health Communication in the Preventions of Alcohol, Tobacco and Other Drugs. Health Educ Q, in press.

Simons-Morton BG, McKenzie J, Stone E, Osganian V, Mitchell P, Strikmiller PK, Ehlinger S, Cribb P, Nader P. Physical activity in a multi-ethnic population of third graders in four states. Am J Public Health, in press.

Simons-Morton BG, Taylor WC, Huang IW. Day-to-day variability in physical activity among third graders. Ped Exerc Sci, in press.

BB

Project Numbers - Annual Report 1996

BIOMETRY BRANCH (BB)

ZO1 HD 00855-05	Statistical Methods for the Common Odds Ratio of a Number of Contingency Tables Kai F. Yu, Ph.D.
ZO1 HD 00856-05	Statistical Methods for a Mixture of Subpopulations Kai F. Yu, Ph.D.
ZO1 HD 00857-05	Methodological Research in Mathematical Statistics and Biostatistics Young J. Lee, Ph.D.
ZO1 HD 00859-05	Meta-Analytic Methods Rebecca DerSimonian, Ph.D.

Z01 HD 00864, 00866-869

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00855-05 BB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Methods for the Common Odds Ratio of a Number of Contingency Tables

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
Others:	A.A. Herman	Visiting Scientist	EB, DESPR, NICHD
	J.F. Troendle	Senior Staff Fellow	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	**PROFESSIONAL:**	**OTHER:**
0.35	0.35	0.0

CHECK APPROPRIATE BOX(ES)

☐ **(a) Human** ☐ **(b) Human** ☒ **(c) Neither**
- ☐ **(a1) Minors**
- ☐ **(a2) Interviews**

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study will develop statistical methods for analyzing categorical data, particularly in the comparison of different groups. Examples are the common odds ratios for a number of contingency tables and the standardized risk difference in the comparison of two groups stratified by a covariate. The optimality properties of the developed methodology will be investigated and established. The likelihood equations are to be derived. The newly developed, maximum likelihood and conditional maximum likelihood methodologies are to be examined. The study has been extended to do a comparison of a number of contingency tables in the form of estimation and hypothesis testing. This methodology is particularly useful in detecting the adequacy of matching in the case-control epidemiological studies. All these methodologies have wide applications to epidemiological studies, biomedical studies, genetics and other disciplines. Examples include testing the Hardy-Weinberg Law across strata in genetics and comparing the black-white differences in birth specific infant mortality curves. The study of the common odds ratio is extended to the case under cluster sampling, where the units within a cluster are not necessarily independent. Theoretical and simulation studies are conducted to validate the proposed methodology.

Project Description:

Objectives: To develop statistical methods for estimating the odds ratio of a number of contingency tables and a family of standardized risk difference parameters. To develop statistical methods for testing hypotheses concerning a number of contingency tables. To study black-white differences in birth weight specific mortality curves. To study the Hardy-Weinberg Law across strata. To develop statistical methods for estimating the common odds ratio under cluster sampling.

Methods Employed: Classical and modern statistical and probabilistic methods are employed to analyze the problems. New methodology is also developed to handle the problems. Computer and numerical techniques are utilized.

Major Findings: An estimator proposed by Greenland and Holland (1991, Biometrics 47, 319-322) for a standardized risk difference parameter is shown to be a maximum likelihood estimator if the consistent estimator of the common odds ratio is appropriately chosen. The statistical problem under consideration is reparameterized for a better understanding. Likelihood equations are derived. A family of easily computable estimators of the common odds ratio of a number of contingency tables is derived from a set of reasonable postulates. A necessary and sufficient condition has been discovered for the strong consistency of this family of estimators. Also, a strongly consistent estimator has been found for the asymptotic variance. Breslow's condition (1981, Biometrika 68:73-84) has been found to be faulty for the consistency of the Mantel Haenszel estimator for large sparse tables. This finding clarifies the confusion over this point in the vast literature. New methodology has been developed to study two sequences of probabilities. It is applied to study the black-white differences in birth weight specific mortality curves. It is also extended to study the Hardy-Weinberg Law across strata. A non-parametric method has been developed to study the common odds ratio under cluster sampling. Asymptotic variance has been obtained. Methodology is also validated by simulation.

Significance to Biomedical Research and the Program of the Institute: Odds ratio and standardized risk difference parameters are commonly used parameters in many biomedical research and epidemiological studies. One example is the Better Babies Project in which the P.I. has participated. This study will enable better understanding and more efficient use of these parameters in substantive research programs. Testing procedures so developed provide more power in comparing black-white differences in birth weight specific mortality curves. They are useful in testing the Hardy-Weinberg Law across strata in genetics. The study of odds ratio under cluster sampling is of significance in survey samples.

Proposed Course of Project: Continuation of development of statistical methodology is planned. Statistical properties are to be investigated. Applications to substantive fields such as genetics will be further examined. Odds ratio under cluster sampling will be further studied. Other kinds of dependence will be examined.

Publications:

Yu KF. A simple comparison of two sequences of probabilities. J Plan Inference 1996. (in press).

Yu KF. On estimating a common odds ratio under binary cluster sampling. [Letter to the Editor]. Stat Med 1996 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00856-05 BB

PERIOD COVERED
October 1, 1995 through September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Statistical Methods for a Mixture of Subpopulations

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
Others:	A.A. Herman	Visiting Scientist	EB, DESPR, NICHD
	H.J. Hoffman	Chief	EB, OD, NIDCD
	R. DerSimonian	Mathematical Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)
Norway National Institute of Public Health (L.S. Bakketeig); National Center for Health Statistics (C.J. Krulewitch); Chung Yuan Christian University (M.S. Yang)

LAB/BRANCH
Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human ☐ (b) Human ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study will examine the intrinsic difficulty of estimating subpopulation characteristics when the identities of the observations are not completely known. Hard clustering and fuzzy clustering techniques to classify observations into subgroups are investigated. This study will develop estimation methodologies for the subpopulation characteristics. The statistical properties of the new methodologies will be studied. The new methodologies will be compared with the existing methodologies designed to remedy the misclassification problem. The new methodologies will alleviate some of the intrinsic difficulties for a heterogeneous mixture of subpopulations. Applications will be made to study the issues concerning biological heterogeneity and measurement error in the birth weight, gestational age and perinatal mortality. This study will also be applied to the investigation of intrauterine growth and pregnancy outcome. Another topic in mixture of distributions is to develop and compare parametric and nonparametric tests to assess homogeneity assumption in mixed models for clinical trials and other biomedical studies.

Project Description:

Objectives: To develop statistical methods for studying subpopulation characteristics in a heterogeneous mixture of subpopulations. To develop parametric and nonparametric tests of homogeneity in mixed models.

Methods Employed: Classical and modern statistical and probabilistic methods are employed. Computer technology is used as a tool for computation and simulation. New methodology is also developed.

Major Findings: Estimation of subpopulation characteristics after a hard clustering of unidentified data into subpopulations is biased and inconsistent as long as there is a chance of misclassification. A notion of fuzzy partition of the data is introduced. It has been established analytically that the developed methodologies of estimation after fuzzy classification is unbiased under some general conditions. The variance of the new methodologies is also analytically established. Simulation studies have been conducted to compare the new methodologies and other existing methodologies and show that the new ones perform much better than the others in many measures. Several tests are developed for binomial, Gaussian and Poisson sampling distributions. They include chi-squared tests, likelihood ratio tests and C-alpha tests.

Significance to Biomedical Research and the Program of the Institute: Estimation after fuzzy clustering has been applied to estimate the perinatal mortality rates for the subpopulations of intrauterine growth retarded and the non-intrauterine growth retarded infants. It can also be used for other pregnancy outcomes. This methodology has also high potential for application in other substantive studies. The tests of homogeneity are important in handling problems of heterogeneity.

Proposed Course of Project: Continuation of development of this methodology is proposed. Complete investigation of the analytic results as suggested by simulation studies is planned. Application to intrauterine growth and pregnancy outcome is to be investigated. Application to study birth weight distribution and gestational age is also planned. To continue the study of mixture in the areas of mixing distributions and distributions of subpopulations.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00857-05 BB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Methodological Research in Mathematical Statistics and Biostatistics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Y.J. Lee	Chief	BMSB, DESPR, NICHD
Others:	J. Troendle	Senior Staff Fellow	BMSB, DESPR, NICHD
	E. Park	Visiting Fellow	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

Hankook University of Foreign Studies (T. Park), Hallym University (S. Jung), ATG (H. Kim)

LAB/BRANCH

Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human ☐ (b) Human ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Methodological research in statistics is motivated often by interest in methodological/theoretical statistical questions. Consulting and collaborative projects also motivate the research because existing methods and theories may be inadequate to handle their data analysis or study design.

Methodological research is ongoing in incomplete data problems, multiple hypothesis testing problem, repeated measure data analysis, missing covariate data problems, and crossover clinical trial problems. Development of new methodologies and innovative new applications of existing methods are focal points of methodological research.

Project Description:

Objectives: To develop statistical methods and theories for the analysis of complex biomedical data and for the design of new biomedical studies.

Methods Employed: Both mathematical methods and computer simulation evaluation methods are applied to develop and evaluate new and existing statistical methods for design, implementation and evaluation of biomedical data analysis.

Major Findings: In biomedical studies, data are often generated in multivariate form and usually incomplete. Namely, we rarely collect a single observation from a study subject. We often repeated a same measurement over time from a study subject. Furthermore, data become incomplete for a number of reasons. For example, in a survival study where the time to death is measured, the patient may survive beyond the observation period or may drop out of the study or may die of causes unrelated to the study outcome. In a repeated measure study, a patient may fail to return for follow-up visits. For example in pregnancy studies where subjects are to make regular clinic visits, some visits are missed and thus, interim pregnancy related variables may be missing. We have also encountered a cross-over clinical trial where subjects are to make a number of follow-up visits and miss some.

Drs. Lee and Park Tae-sung are investigating statistical methods to deal with multivariate repeated measure studies with missing observations and visits. This problem is motivated by the NICHD tubal ligation study where the effect of tubal sterilization on estrogen and progesterone during luteal period is measured over time.

In repeated measures studies, the main interest is often lies in comparing group effects. The comparison of group effects can usually be performed by testing the equality of group means. Sometimes groups are formulated in an order relation. Dr. Park Tae-sung proposed a test procedure for testing the equality of group effects. Group effects can be estimated by means and can be tested by the LRT statistics. The results have been summarized into a paper and will appear in the September issue of Biometrika. Second, Dr. Park has studied cases where responses follow a quasi-likelihood formulation such as Poisson and binary outcomes. He and his coworkers proposed a test based on the Wald statistics. The results have been summarized into a paper which has been submitted for publication.

In repeated measures studies, missing data commonly occur. Dr. Park Tae-sung and his co-worker proposed a model which can be used to investigate the missing data mechanisms. They mainly focus on the model using the GEE approach. Through simulations studies, they show that the proposed test has a reasonable size and a good power for a moderate sample size. The results have been summarized into a paper and has been accepted by Statistics in Medicine.

In simulation studies for repeated measures data, it is required to generated correlated variates. Drs. Park Tae-sung and his coworkers proposed a new algorithm to generate correlated binary variates. Their method is simple and easy to implement. The key idea is to use independent Poisson variables and to define correlated variables as sum of those independent variables. The results have been summarized into a paper which will appear in the November issue of American Statistician.

Drs. Lee and Jung have been working on the logistic regression method for the survival data with censored observations. They have produced a paper which is accepted for publication in the Journal of American Statistical Association with subject to a minor revision. They now apply the method to predict the survival for given covariates, and compare their method to the well known Cox regression method in predicting the survival probability. They also study how their method can be applied in testing the proportional hazard model assumption which is a requirement for the Cox regression method.

Dr. Lee developed a nonparametric method for missing data and presented the paper in the Richmond ENAR meeting. He has also developed a method for adjusting for missing data in clinical trials and submitted a paper.

In crossover clinical trials with repeated measurements where some observations are missing, the parametric split-plot method excludes subjects with missing observations. However, when the number of subjects with missing observations is substantial, the parametric approach will cause loss of efficiency. Drs. Lee and Kim have proposed a nonparametric method based on ranks of observations within a subject. This research was motivated the NICHD osteogenesis imperfecta study where subjects were crossed of between two treatments and followed during each treatment period. A paper has been presented at the Richmond ENAR meeting.

In the DIEP study, blood samples were drawn from regularly scheduled weekly return visits. One of the questions was to determine whether weekly values of some blood chemistry are related to the fetal loss within the first trimester. However, many blood chemistry observations are missing due to missing visits, causing difficulty in analyzing the data. Analyzing complete data only will not only introduce bias into the analysis but also make the data analysis lose efficiency. Drs. Lee and Kim are investigating multiple imputation methods to make up for the missing covariates. A paper will be presented at the Chicago Annual Statistics Meetings.

Dr. Troendle has been working on constructing a permutational step-up testing procedure for adjusting p values for multiple endpoints. Step-up tests are more powerful than step-down tests in certain situations. Although the algorithm derived for step-up adjusted p values is more computational than the existing step-down algorithm, some modifications have been made to reduce the computation. A paper has been accepted for publication in Biometrics.

A simulation study of the properties of step-down permutation tests has been completed by Dr. Troendle and Clifford Blair. It was found that the step-down tests are particularly powerful when many endpoints are involved, the data are significantly correlated, or distributional assumptions are questionable. The paper has appeared in Statistics in Medicine, Vol. 15, 1107-1121.

When multiple endpoints are involved, closed testing algorithms in conjunction with global tests have been proposed for use in identifying significant outcomes. An alternative is to use a stepwise assessment of the endpoints, where at each step an adjustment is made for the endpoint left. Drs. Troendle and Legler have completed a comparative study of some new and some existing methods for small sample sizes, and a paper has been submitted for publication.

Dr. Troendle has discovered the most powerful permutation test among all similar tests against a simple normal alternative in the multivariate two sample location shift problem. Unfortunately, no most powerful test exists for composite hypotheses. A simple approximation yields a new test which is applicable to composite one sided tests. The new test has been compared to Hotelling's T^2 permutation test, Boyett and Shuster's maximal t test, and to the approximate likelihood ratio test of Tang et al. A paper has been submitted for publication.

Along with Cliff Blair, Deborah Rumsey and Pamela Moke, Dr. Troendle has written a paper on comparing several treatments to a control when treatment is expected to increase variability. When the affect of treatment includes a variance inflation, the usual F test loses power and a modification has been proposed. The paper compares the power of the known methods to a likelihood ratio test derived for this scenario for several types of data. The methods are illustrated on some analyses of optic neuritis data.

Drs. Troendle, Yu, and Herman have been working on standardization systems for birth weight and gestational age when the outcome measure is infant mortality. The analysis compares an hoc method of grouping infants into simple categories of birth weight (Wilcox, 1979), to a new procedure which extends the Wilcox method to incorporate gestational age while removing much of the ad hoc methods employed in that work. The method provides a decomposition of the infant mortality for a population into two sources: that expected for a standard population with a bivariate Gaussian distribution of birth weight-gestational age pairs centered at the population means and having the same birth weight-gestational age specific mortality, and the additional mortality obtained because of the actual skewed distribution of births. Applied to U.S. data, the new method shows a contrast to the conclusions of Wilcox and Russell(1986).

Dr.Troendle has been working with Colin Begg to find a proper analysis of the interim data for the Growth Hormone trials. The ongoing trials use variable starting ages, variable follow-up periods, and have the primary endpoint of adult height. The definition of adult height is a complicating factor, but even supposing a fixed age at which height can be taken to represent adult height the analysis is challenging. To incorporate the majority of patients who either are still too young for adult height to have been obtained or who dropped out, we have used the EM algorithm to estimate the mean height at age 16 for each treatment group. The next problem is in obtaining estimates of the standard errors for the estimated means. The usual estimate is inappropriate because of the missing data. This work is still being pursued.

Significance to Biomedical Research and the Program of the Institute: All the methodological researches are motivated by data analysis problems encountered in the course of collaborative and consultative activities. We will be better able to analyze biomedical data and design biomedical studies.

Proposed Course of Project: Continue to explore and develop statistical methods applicable to biomedical data and projects.

Publications:

Blair RC, Troendle JF, Beck RW. Control of family wise errors in multiple endpoint assessments via stepwise permutation tests. Stat Med 1996;15:1107-12.

Kimonis VE, Troendle J, Rose, SR, Yang ML, Markello TC, Gahl WA. Effects of Early Cysteamine Therapy on Thyroid Function and Growth in Nephropathic Cystinosis. J Clin Endocrinology Metabolism 1995;80: 3257-61.

Park CG, Park T, Shin DW. A simple method for generating correlated binary variates. American Statistician, in press.

Park T, Shin MW. A practical extension of generalized estimating equations approach for longitudinal data. Communications in Statistics-Theory and Method 1995;23:1547-64.

Read JS, Troendle JF, Klebanoff MA. Infectious Disease Mortality Among U.S. Infants, American J Public Health, in press.

Shin DY, Park CG, Park T. Testing for ordered group effects with repeated measurements. Biometrika, in press.

Troendle JF. A Permutational Step-Up Method of Testing Multiple Outcomes. Biometrics, 1996;52:125-38.

Troendle JF, Yu KF, Herman AA. A Scale Problem to Avoid. A J Public Health, in press.

Westhoff C, Gentile G, Lee YJ, Zacur H, Helbig D. Predictors of ovarian steroid secretion in reproductive age women. American J. Epid, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00859-05 BB

PERIOD COVERED
October 1, 1995 to September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Meta-Analytic Methods

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Rebecca DerSimonian	Mathematical Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH
Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.60	0.60	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human ☐ (b) Human ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project consists of four components which continue from last year:

The first develops and compares parametric and non-parametric tests to assess the assumption of homogeneity of effects in data from a series of trials. This component considers data from Gaussian, binomial, and Poisson sampling distributions. Preliminary results from this component is submitted to Statistics in Medicine.

The second considers meta-analytic methods for combining the evidence from a series of HIV seroprevalence studies to estimate HIV prevalence in a target population. Sentinel studies are typically investigations of incompletely defined cohorts which are convenient to survey but are often based on non-probability samples. Self-selection and similar issues inherent in sentinel studies makes generalizing results from a single study to a target population problematic. This research assesses the use of formal meta-analytic methods for HIV prevalence estimation in a target population by incorporating information from several HIV sentinel seroprevalence studies. Preliminary results from this component appeared in Annals of Epidemiology 1995;5:447-54.

The third component addresses issues that pertain to the use of meta-analysis in the design and monitoring of clinical trials. This research evaluates the role of formal incorporation of external evidence summarized from a meta-analysis of previous or concurrent results into sample size considerations and stopping rules during the conduct of a clinical trial. Preliminary results from this component is appeared in Statistics in Medicine 1996;15:1237-48.

Project Description:

Objectives: To develop and refine statistical methods for combining data from a series of clinical trials and HIV seroprevalence sentinel studies.

Methods Employed: For meta-analysis of clinical trials, this project considers fixed and random effects as well as Bayes models for meta-analysis of data under various heterogeneity assumptions and several scales of measurement, including the risk difference and the odds ratio as measures of effect. Both parametric and non-parametric methods are employed for assessing homogeneity of effects. The role of meta-analysis in the design and monitoring of a new clinical trial is also considered.

For meta-analysis of HIV seroprevalence studies to estimate prevalence in the target population, this project considers empirical Bayes methods to incorporate information from related sentinel studies. This research characterizes the special epidemiologic and statistical issues inherent in sentinel studies for HIV prevalence estimation and develops methodology that addresses them when incorporating the information from several such studies.

Major Findings: For meta-analysis of clinical trials, several tests of heterogeneity for binomial, Gaussian, and Poisson sampling distribution are developed and compared under various heterogeneity assumptions (submitted to Statistics in Medicine). Furthermore, the impact of using meta-analytical results in the design and monitoring of the NICHD trial on preeclampsia is evaluated Statistics in Medicine 1996;15:1237-48).

For meta-analysis of seroprevalence studies, we find that prevalence in an individual HIV sentinel serosurvey is time-averaged and vulnerable to several time-dependent biases and that self-selection and laboratory methods are additional likely sources of bias. Incorporating the information from several such HIV sentinel seroprevalence studies mitigates the possible impact of these biases inherent in the individual studies (Annals of Epidemiology 1995;5:447-54).

Significance to Biomedical Research and the Program of the Institute: The statistical methods developed in this project are applicable to many data sets relevant to the Institute where the evidence on a particular topic is conflicting and/or the sample sizes in the available studies are too small to yield unequivocal results. An example of the applicability of the methods to relevant problems in the Institute is the workshop organized by the Division on "The Role of Meta-Analysis in the Design and Monitoring of Clinical Trials." Being directly relevant to this topic, preliminary results from this project were presented at the workshop, which was prompted by concerns raised at the Data Safety and Monitoring Board of the large multi-center trial conducted by the Institute on "Calcium Supplementation for the Prevention of Preeclampsia (CPEP)."

Another example is the presentation made at the CPEP Steering Committee and a subsequent invitation to do a similar presentation at the meeting of The International Society for the Study of Hypertension in Pregnancy in Pregnancy. Recent reviews (and accompanying editorials) published in JAMA on calcium supplementational for the prevention of hypertensive disorders in pregnant women. The negative results from CPEP which have not yet been disseminated, are in direct conflict with these conclusions. The presentation focuses on critical evaluation of the reviews claiming calcium efficacy and interpretation of published data about calcium efficacy from a proper quantitative perspective. Contrary to the previous reviews, the conclusions from proper meta-analysis of the data on this topic turn out to be quite consistent with the results of CPEP.

Proposed Course of Project: Continue to explore and develop the methodology for tests of homogeneity, particularly the non-parametric ones, to further evaluate the role of meta-analysis in planning a new clinical trial, and to develop methods for combining data from HIV sentinel studies comparing the results with those from back calculations.

Publications:

Strickler H, Hoover DR, DerSimonian R. Problems in interpreting HIV sentinal seroprevalence studies. Ann Epidemiol 1995;5:447-54.

DerSimonian R. Meta-analysis in the design and monitoring of clinical trials. Stat Med 1996;15:1237-48.

CSB

Project Numbers - Annual Report 1996

COMPUTER SCIENCES BRANCH (CSB)

ZO1 HD 01802-06 Data Coordinating Center for the NICHD Study of Early Child Care
PRJT. COMPLETED (10/1/95-2/14/96) Ann C. Trumble

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01802-06 CSB

PERIOD COVERED

October 1, 1995 through February 14, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Data Coordinating Center for the NICHD Study of Early Child Care

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A.C. Trumble Computer Specialist CSB, DESPR, NICHD

COOPERATING UNITS (if any)

CRMC, NICHD/NIH

LAB/BRANCH

Computer Sciences Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human ☐ (b) Human ☐ (c) Neither

- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study is a 10-site cooperative agreement to examine the effects of non-maternal care, especially during the first year of life. It focuses on the social and intellectual development of children in an ecological framework which takes into account the complex interactions of non-maternal care experiences with home and family conditions, parenting practices, and child characteristics. Over 1200 families were enrolled in the study during 1991. These families will participate in the study for 36 months.

The Data Coordinating Center (DCC) was responsible for data management and protocol monitoring for Phase I. It served as the focal point for receipt of collected data and for data processing.

The DCC completed processing 24 and 36 month data. The data were entered into computer files, edited and summarized for monitoring purposes. Routine edit reports were distributed to the sites regularly. Project monitoring reports were distributed to the Steering Committee quarterly.

For close-out procedures of data monitoring for Phase I, the DCC sent all data collection instruments, related materials, and tapes containing over 1,000 archived datasets, to the Phase II Data Acquisition and Analysis Center. The remaining Phase I analysis data sets were sent to the Phase I Analytic Section. A final report was delivered.

Project Description

Objectives: To examine the effects of non-maternal care, especially during the first year of life on the social and intellectual development of young children. The Data Coordinating Center was located in the Computer Sciences Branch of the Division of Epidemiology, Statistics and Prevention Research. The objective of the Data Coordinating Center is to assure that the protocol is uniformly administered at all sites. This includes the preparation of Manuals of Operations; management of monitoring subject recruitment; design of data collection instruments, data entry methods and data base management systems; and generation of statistical reports.

Methods Employed: At each of the ten sites participating in the study, approximately 120 infants and their parents were enrolled in this study shortly after the infant's birth and will be followed until the infant is 3 years old. Over the 36 months of participation, the infant was visited and observed in the home, in child care, and in a laboratory setting.

Through close monitoring of the sample recruitment, a sample has been selected which closely represents the goal. This study is being conducted in collaboration with the Center for Research for Mothers and Children.

Major Findings: Infants with extensive child care experience did not differ from infants without child care in the distress they exhibited during separations from their mothers. There were not significant main effects of child care experience (quality, amount, age of entry, stability, or type of care) on attachment security or avoidance. Infants were least likely to be secure when their mothers were insensitive and they were in poor child care arrangements. Infants were also less likely to be secure when their mothers were insensitive and they experienced more than minimal amounts of child care, or more than one child care arrangement. In addition, boys experiencing many hours in care and girls in minimal amounts of care were somewhat less likely to be securely attached.

Significance to Biomedical Research and the Program of the Institute: This project will help determine the extent and type of non-maternal care used in this country and the effects that different patterns of care have on the development of children in the first three years of life.

Proposed Course: The data collection for phase I is complete. The Data Coordinating Center for phase II is being carried out under a grant.

Publications:

Phillips D, Huston A and the NICHD Early Child Care Network. Poverty and patterns of child care. In: Brooks-Gun J & Duncan G, eds. Consequences o growing up poor. New York: Russell-Sage. (in press)

Vandell D and the NICHD Early Child Care Network. Characteristics of infant child care: Factors contributing to positive caregiving. Early Childhood Research Quarterly. (in press)

DESPR

Project Numbers - Annual Report 1996

DIVISION OF EPIDEMIOLOGY, STATISTICS AND PREVENTION RESEARCH (DESPR)

Z01 HD 01703-07	Study of Pregnancy Outcome, Maternal Death and Child Health in Pakistan Heinz W. Berendes, M.D., M.H.S.
Z01 HD 01706-02	A Randomized Controlled Trial for the Evaluation of a New Antenatal Care Model Heinz W. Berendes, M.D., M.H.S.

Z01 HD 01707-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01703-07 ESPR

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Pregnancy Outcome, Maternal Death and Child Health in Pakistan

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: H.W. Berendes Director DESPR, NICHD

COOPERATING UNITS (if any)

Department of Community Health, Aga Khan University (Drs. Joseph McCormick, Fariyal Fikree, Farid Midhet, and Mehtab Karim), Special Program for Research, Reproduction and Family Planning of the WHO (Dr. Jose Villar)

LAB/BRANCH

Office of the Director, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human ☐ (b) Human ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has three components.

The Maternal and Infant Mortality Survey (MIMS) will provide estimates of maternal and infant mortality rates from population based samples in Karachi and the four provinces of Pakistan. In addition, the Survey will provide information on causes of maternal and infant deaths and demographic and other determinants of maternal and infant mortality.

The Pregnancy Outcome Study (POS) is a cross sectional and prospective study of pregnant women from selected katchi abadies to identify determinants of poor birth outcome in this population.

The Child Survival Study (CSS) is a follow up study of live born children from POS through two years of age to assess mortality, morbidity and physical and developmental measures.

A new component is the development of an intervention to reduce infant mortality and morbidity by training all levels of care providers (dais, lady health visitors, midwives, physicians) to recognize timely high risk conditions during pregnancy or at delivery, set up a referral and transportation system and get high risk women timely referral to a tertiary medical facility.

Project Description

Objectives: Based upon the findings on the Maternal and Infant Mortality Surveys an intervention is being developed in Karachi to reduce maternal and infant mortality. It is proposed to conduct a community-based trial to evaluate the effectiveness of an intervention strategy which will consist of training, monitoring and supervision of care providers, setting up a referral and transportation system to tertiary care providers of high risk women and women with complications around the time of labor and delivery and an information, education and communication campaign to increase awareness at the community level.

Methods Employed: The Maternal and Infant Mortality Survey (MIMS) is a two step survey. The first step consists of an enumeration of household composition over the last five years and identification of any deaths occurring during that period. The second step is a detailed interview (verbal autopsy) of household members to identify pregnancy related deaths, to obtain information about possible cause of maternal deaths, as well as of infant deaths. The survey in Karachi will be based upon sites which are currently part of a primary health care network developed by the Department of Community Health of the Aga Khan University and will include approximately 10,000 households. There will additionally be surveys done in each of the four provinces, that is, Baluchistan, Sindh, Northwest Frontier Territory, and the Punjab. Sample sizes for the provincial surveys will vary between 10-20,000 households depending on availability of funds. Cluster samples will be taken from select sites of each province.

POS will identify all pregnant women at a given time in the katchi abadies which are part of the primary health care system of the Aga Khan University Department of Community Health and follow them to delivery to identify determinants of poor birth outcome. Information is collected through questionnaire and by physical examination. Since most women deliver at home attended by traditional birth attendants, a notification system is in place notifying the project about any births within two days to obtain birth weight as well as modified Dubowitz evaluation to differentiate preterm births from intrauterine growth retardation among low birth weight children. The Pregnancy Outcome Study is completed.

CSS is a prospective follow up of live births from POS to age two to assess mortality and morbidity and determinants of physical growth and development. The follow up will include interviews, physical examination, measurements of physical growth, as well as behavioral assessments. The Child Survival Study is completed.

It is proposed to implement an intervention in a section of Karachi called Korangi consisting of ten contiguous wards with a population of approximately 500,000. As controls, another site is chosen nearby of similar size and similar population characteristics. Both intervention and control sites will be subjected to a pre- and post-intervention survey to obtain information on current maternal and infant mortality rates and complications around the time of pregnancy. This project is still under development because of delays in funding.

Major Findings: The MIMS Surveys have been completed now. While the overall maternal mortality rate is high in all sites, there is considerable variation between sites, the lowest rate is in Karachi and was 2.7/1000 live births and the highest in parts of Baluchistan of about 12/1000. Infant mortality rates vary accordingly, also from a low of about 75/1000 to a high of 210/1000 live births. This range represents different katchi abadies within the city of Karachi.

Considerable progress has been made in the analysis of data from the Pregnancy Outcome Study. Current analyses deal with risk factors related to intrauterine growth retardation which is a major risk factor in developing countries and accounts for the major fraction of low birth weight births in developing countries. Analysis of the data suggests that major risk factors include socioeconomic conditions, including housing construction, paternal and maternal education, paternal employment and the source of water supply, as well as ethnic differences apparent among the different ethnic groups in these communities which consists of Hindus, Muslims and Christians. Biological factors related to the risk of IUGR include young maternal age, primiparity and high parity, history of poor prior pregnancy outcome, a short interconception interval, low maternal height and weight, as well as a low mid-arm circumference and low skinfold thickness reflecting maternal nutritional status as well as a non-vegetarian diet during pregnancy. In addition, there was a marked increase in risk due to consanguinity.

In a multi-variate analysis, remaining risk factors included the source of water supply, maternal education and paternal employment, primiparity and grand multiparity, consanguinity, less than one year interval between birth to conception, low maternal height and weight and also non-vegetarian diet. These findings suggest possible intervention especially related to improved nutrition but also raising the status of women by mandating primary education of all school age girls.

In an analysis of the contextural determinants of maternal mortality in rural districts of Pakistan, we used data about the availability of health services, clinics, physicians and lady health visitors, as well as modes of transportation and distance to such facilities as contextural variables which may impact upon maternal mortality. An understanding of such variables on mortality is of key importance in the design of appropriate interventions.

It is quite clear that in order to deal with complications of pregnancy and child birth and to prevent maternal deaths access to a well equipped hospital is necessary. In addition, for the most part, many of these complications related to maternal mortality are not easily predictable nor preventable. Therefore, access to adequate obstetrical care is the essential intervention to reduce maternal deaths in developing countries. Not unexpectedly, distance is a major determinant of mortality and pregnant women at high risk of maternal mortality living in a remote village are more likely to die than pregnant women at comparable risk but closer to a medical facility. There also is not unexpectedly a relationship between the ratio of population per primary health care facility indicating that the increase in population per primary health care facility is associated with an increase in maternal mortality. Unfortunately we found little evidence of any role of government health services currently in place in preventing maternal deaths in the areas of Baluchistan and Northwest Frontier Providence included in the analysis. The main conclusion from this analysis is that the mortality rate of mothers is lower in areas which are located closer to a large city which have developed better roads and communication systems.

Using data from the Child Survival Project, we completed an analysis with variables related to stunting in children from birth to two years of age. In this particular study, approximately 1000 children were followed from birth to 24 months and seen at 1 month, 3 months, 6 months, 9 months, 12 months, 18 months and 24 months. Information was obtained at each visit about intercurrent illness, especially episodes of diarrhea and of upper and lower respiratory tract infection, and infant feeding practices, and also measurements were taken of the child's height and weight and head circumference at each one of these intervals. Not unexpectedly, the rate of stunting in this population is high and increases from a level of 27 percent to 52 percent at 12 months. Wasting is relatively low with a rate of 2 percent at 3 months, increasing to 6.6 percent at 12 and 24 months. In a uni-variate analysis, the risk of stunting was increased by low level of income, and poor building material used for the house construction. Also, lack of water in the house increased the risk of stunting, as did lack of electricity. Other factors related were crowding and consanguinity. High maternal and paternal education was protective. Both maternal height and weight were related to stunting with lower maternal height and weight increasing the risk of stunting. Stunting was lower among primigravidas. There was a strong correlation between IUGR status at birth and risk of stunting.

Breast feeding, either exclusive or partial breast feeding, was protective at 6 and 12 months on the rate of stunting.

There was a limited effect of intercurrent illness on stunting. An illness index consisting of frequency of diarrhea, upper and lower respiratory infection and skin rash showed an increase risk of stunting related to illness as compared to those without intercurrent disease.

In a multi-variate model, significant effects are seen for income, water supply and maternal education. Of maternal variables, low maternal weight increased the risk and primiparity decreased the risk of stunting. Prior IUGR status at birth stayed in the model and increased the risk of stunting significantly. The occurrence of diarrhea at 6 months increased the risk with no effect seen at subsequent intervals.

The intervention is currently under development and will begin in 1997.

Significance to Biomedical Research and the Program of the Institute: The identification of determinants of maternal and infant mortality is a major research interest of the Institute and the findings of this project have specific public health importance to the country of Pakistan and other countries who are in a similar stage of development. A conference was held in the spring of 1994 to present the findings from the Maternal and Infant Mortality Surveys to an audience consisting of representatives of Ministries of Health, the Provincial

Government, leading academicians and representatives of international health agencies for the purpose of acquainting this audience to the findings from the surveys but also as a means of developing possible interventions to address the high maternal and infant mortality. The World Bank participated in the planning for this conference and underwrote some of the costs. The proceedings of the conference are in preparation for publication.

The interventions to reduce maternal and infant mortality in developing countries is clearly of major public health significance and of interest to this Institute.

Proposed Course: The intervention is currently under development with active participation of the Director of the Division. Funding is provided by the World Bank and by AID with some funding expected also from UNICEF in Pakistan. The Director, DESPR will serve as a senior technical advisor in this project with active participation in its design and implementation as well as the analysis of the data.

Publications:

Fikree FF, Gray RH. Demographic survey of the level and determinants of perinatal mortality in Karachi, Pakistan. Ped Perinat Epid 1996;10:86-96.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 01706-02 ESPR

PERIOD COVERED
October 1, 1995 through September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
A Randomized Controlled Trial for the Evaluation of a New Antenatal Care Model

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	H.W. Berendes	Director	DESPR, NICHD, NIH

COOPERATING UNITS (if any)
WHO, Geneva (J. Villar); Rosario Centre for Perinatal Research, Rosario, Argentina (J.M. Belizan); Jehda, Saudi Arabia (R. Al-Mazrou); Havana, Cuba (U. Farnot); Congella, South Africa (J. Moodley); Khan Kaen, Thailand (P. Lumbiganon)

LAB/BRANCH
Office of the Director, DESPR

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human ☐ (b) Human ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This is a randomized controlled clinical trial which compares a revised system of prenatal care to a conventional prenatal care system currently in place in five different cities in different developing countries. Expert review in this country, Sweden and elsewhere of the content and visits schedule for prenatal care has indicated that there is little scientific evidence which supports the current schedule of visits as well as its content. Several experts and national groups have therefore recommended to limit the number of prenatal visits for low risk women in order to reduce costs and to save resources to be used for high risk women. These same groups have also recommended trials to evaluate different models of prenatal care. Since prenatal care is viewed as a major determinant of infant mortality and morbidity, this is a topic of major research interest to the Institute.

The WHO through a technical advisory panel has developed a modified antenatal care model which substantially changes the content of prenatal care and allows a schedule of four visits for low risk women spread across the prenatal period. The emphasis in the revised model is on the recognition and diagnosis of conditions and complications of pregnancy known to affect the health of women and/or the fetus and specific interventions and treatments of such conditions and complications once identified. The DESPR, through its Director, has been a member of the Technical Advisory Panel which designed the new model of prenatal care, has served as the Chair of the Technical Advisory Panel of the WHO, and is currently serving as Chairman of the Steering Committee.

The new prenatal care model will be evaluated in five sites by randomizing clinics in each of these five sites to the current system of prenatal care or the new proposed model of prenatal care.

Project Description

Objectives: To conduct a randomized clinical trial comparing two models of antenatal care to establish the relative merits of each model. It is intended to test whether the proposed new model is as effective as the traditional multi-visit model with regard to maternal mortality and perinatal morbidity and mortality, and satisfaction. This trial tests the hypothesis whether the new model of prenatal care is equivalent in terms of certain primary outcomes to the current system in place. Primary outcomes are the rate of low birth weight and the rate of a maternal mortality indicator index consisting of the presence of at least one of the following: proteinuric-preeclampsia or eclampsia (during pregnancy or within 24 hours of delivery); postpartum anemia (less than 90 grams per liter); severe urinary tract infection/polynephritis (requiring antibiotic treatment or hospitalization). In addition a number of secondary outcomes have been identified and will be tested in the analysis.

Methods Employed: This trial will be conducted in five sites consisting of Rosario, Argentina; Havana, Cuba; Congella, South Africa; Khan Kaen, Thailand; and Jehda, Saudi Arabia. Randomization will be by clinics or other units of prenatal care providers and the clinics will be assigned to the current regimen of prenatal care or the new model to be tested. There will be 12 units or clinics per site and the patient population included in this trial across the five sites will be between 30-36,000. The individual clinics or units of randomization are chosen to be similar in numbers of patients attending, and characteristics such as race, ethnicity, religion, socioeconomic status, and health, as well as rates of various perinatal outcomes. The new prenatal care system administered over four visits will include only activities which have been demonstrated to be effective in improving pregnancy and maternal, as well as newborn outcomes. They are specific interventions included in each of the four visits related to specific adverse health conditions identified. In addition, health education information for the mother are the central part of every visit, emphasizing the danger signs of complications during pregnancy and actions that must be taken. The protocol, in a very direct manner, indicates what tests must be done at every visit and what actions must be taken for different results of such tests.

Sample size estimates are based on the anticipated effect of prenatal care on the rate of low birth weight as well as the rate of maternal morbidity as defined for this project. Assuming that each of these two indicators will have on the average an incident of about 100 per 1000 live births in the participating clinical centers, the study is designed to detect an 18 percent change.

Baseline surveys of characteristics of the current population and current practices have been done in two of the sites (Cuba and Thailand) and are currently conducted in Jehda, Saudi Arabia and Rosario, Argentina, and will be done later this fall in Congella, South Africa. The antenatal care trial has started to enter women in January in Thailand and Cuba; and Argentina and Saudi Arabia will follow with South Africa later in the year.

It is expected that recruitment of patients for this trial will take approximately 18 months and if this is a realistic estimate, data would be available for analysis approximately 2 to 2 1/2 years from now. Thus the study should be completed within three years from now.

Major Findings: None

Significance to Biomedical Research and the Program of the Institute: To test new models of prenatal care is of major research interest and public health importance in the field of maternal and child health. Clearly if it could be shown that a revised model of prenatal care consisting of four visits and focusing on conditions of pregnancy known to affect outcome is equivalent in outcome to what is currently in place, this should result in a major saving of resources which could then be used for dealing with high risk pregnancies.

Proposed Course: The trial has started in Cuba and Thailand in January and other sites will join during the summer and fall of this year. Trial results should be available late in 1998 or early 1999.

The Division supports the Rosario site in Argentina through a contract. Funding for the other sites is provided by different donor agencies and WHO.

Publications: None

EB

Project Numbers - Annual Report 1996

EPIDEMIOLOGY BRANCH (EB)

ZO1 HD 00331-13	Diabetes in Early Pregnancy (DIEP) James L. Mills, M.D., M.S.
ZO1 HD 00334-13	Low Birth Weight Across Generations Mark A. Klebanoff, M.D., M.P.H.
ZO1 HD 00361-10	Child Health Supplement to the 1988 National Health Interview Survey Mary D. Overpeck, M.P.H.
ZO1 HD 00369-08	Adverse Perinatal Events and Subsequent Injury-related Death Mark A. Klebanoff, M.D., M.P.H.
ZO1 HD 00373-08	Calcium Supplementation in Pregnancy for the Prevention of Preeclampsia Richard J. Levine, M.D.
Z01 HD 00379-07	Data Analysis from the Vaginal Infections and Prematurity Study Mark A. Klebanoff, M.D., M.P.H.
Z01 HD 00383-06	Analyses of Data from the Collaborative Perinatal Project Mark A. Klebanoff, M.D., M.P.H.
ZO1 HD 00384-04 **TERMINATED**	Field Trial of Oral Cholera Vaccines in Bangladesh John D. Clemens, M.D.
ZO1 HD 00385-04 **TERMINATED**	Epidemiology of Rotavirus Infections in Bangladesh John D. Clemens, M.D.
ZO1 HD 00386-05	Evaluation of a Water-Sanitation Intervention in Egypt John D. Clemens, M.D.
ZO1 HD 00389-04 **TERMINATED**	Studies of the Epidemiology of Pediatric Shigellosis in Bangladesh John D. Clemens, M.D.
ZO1 HD 00392-05	Fatal Injuries to U.S. Infants, 1983-87 Ruth A. Brenner, M.D., M.P.H.
ZO1 HD 00801-21	Studies Based on the Medical Birth Registries of Norway and Sweden Allen A. Herman, M.D., Ph.D.
ZO1 HD 00861-14	Assessment of In-Utero Fetal Growth Patterns in Relation to Outcome at Birth Allen A. Herman, M.D., Ph.D.
ZO1 HD 00872-11	Factors Associated with Premature Births: Missouri Follow-Back Survey Allen A. Herman, M.D., Ph.D.
Z01 HD 2500-04	ETEC Epidemiology and Vaccine Research in Lower Egypt John D. Clemens, M.D.

Continued →

EPIDEMIOLOGY BRANCH (EB)
(continued)

Z01 HD 2502-04	NICHD-Health Research Board of Ireland Neural Tube Defects Study James L. Mills, M.D., M.S.
Z01 HD 2506-04	Lack of Age-Appropriate Immunizations Among Infants Born in D.C. Ruth A. Brenner, M.D., M.P.H.
Z01 HD 2507-04	Prostaglandin Excretion in Preeclampsia James L. Mills, M.D., M.S.
Z01 HD 2510-03 **TERMINATED**	Diet, Maternal Nutritional Status, Blood Pressure and Fetal Growth Nebiat Tafari, M.D.
Z01 HD 2511-02 **TERMINATED**	Immunogenecity of Routine Childhood Vaccines in HIV Positive Children Jennifer S. Read, M.D.
Z01 HD 2512-03	Birth Certificate Linkage to Growth and Health Measures Using NHANES III Mary D. Overpeck, Dr. P.H.
Z01 HD 2513-03	Survival Status and Risk Factors Analysis of Premature High Risk Deliveries Mary D. Overpeck, Dr. P.H.
Z01 HD 2514-03	Childhood Drowning Deaths - A National Analysis of Death Certificate Information Ruth A. Brenner, M.D., M.P.H.
Z01 HD 2515-03	Prevention of Childhood Injuries - Phase I Injury Surveillance Ruth A. Brenner, M.D., M.P.H.
Z01 HD 2516-03	The Role of Bathtub Rings and Seats in Infant Drowning Deaths Ruth A. Brenner, M.D., M.P.H.
Z01 HD 2517-02 **TERMINATED**	First Week Changes in Birthweight and Other Neonatal Anthropometric Parameters Nebiat Tafari, M.D.
Z01 HD 2518-02 **TERMINATED**	Effectiveness of Rapid Plasma Reagin Screening in Gestational Syphillis Nebiat Tafari, M.D.
Z01 HD 2519-02	Determination of Protection Level of Maternal Antibody to Group B Streptococcus Feng-Ying Lin, M.D., M.P.H.
Z01 HD 2520-02	Maternal Caffeine Use in Pregnancy Outcome Mark A. Klebanoff, M.D., M.P.H.
Z01 HD 2521-02	Replacement of Pregnancy Losses: Interpregnancy Interval in Adolescence Yvette R. Johnson, M.D., M.P.H.
Z01 HD 2522-02	Intrapartum Antibiotic Prophylaxis Against Early-Onset Group B Streptococcal Disease Yvette R. Johnson, M.D., M.P.H.

Continued →

EPIDEMIOLOGY BRANCH (EB)
(continued)

Z01 HD 2523-02	Studies Based on Maternally Linked Birth Registries of Missouri and Utah Allen A. Herman, M.D., Ph.D.
Z01 HD 2524-02	Occupational Nonfatal Injuries in U.S. Children Mary D. Overpeck, Dr. P.H.
Z01 HD 2525-02	World Health Organization Study of Health Behavior in School Children (WHO-HBSC) Mary D. Overpeck, Dr. P.H.
Z01 HD 2526-01	Survival and Health Status Analysis of Premature, High-Risk Deliveries Mary D. Overpeck, Dr. P.H.
Z01 HD 2527-01	Fetal Growth in Chronically Malnourished Women Yvette R. Johnson, M.D., M.P.H.
Z01 HD 2528-01	Epidemiology of Typhoid Fever in India John D. Clemens, M.D.
Z01 HD 2529-01	Field Trial of Killed Oral Cholera Vaccines in Vietnam John D. Clemens, M.D.
Z01 HD 2530-01	Economic Analysis of Killed Oral Cholera Vaccine in Refugee Settings in Malawi Abdollah B. Naficy, M.D.

Z01 HD 002531-2600

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00331-13 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Diabetes In Early Pregnancy (DIEP)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.L. Mills	Chief, Pediatric Epidemiology Section	EB, DESPR, NICHD
Other:	M.R. Conley	Computer Specialist	EB, DESPR, NICHD
	Y.J. Lee	Chief	BMSB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Cornell Univ. Med. Center, NY (L. Jovanovic); Brigham and Womens Hosp., Boston, MA (L. Holmes); Northwestern Univ. Med. Center, Chicago, IL (J.L. Simpson); Univ.of Pittsburgh, Pittsburgh, PA (J. Aarons); Univ. of Washington, Seattle, WA (R. Knopp)

LAB/BRANCH
Epidemiology Branch

SECTION
Pediatric Epidemiology Section

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Diabetes in Early Pregnancy Project was designed 1) to examine the relationship between maternal diabetic control during organogenesis and malformations in the offspring, and to identify, if possible, a specific teratogenic factor or factors in the diabetic metabolic state; and 2) to compare early fetal loss rates in women with diabetes and in non-diabetic control subjects. We found that diabetic women who came into care before the period of organogenesis achieved better results than those who came in later; but their results were still poorer than for non-diabetic control subjects. Differences in maternal glucose levels during organogenesis did not explain the malformations in the offspring of the women who were followed throughout pregnancy. These results suggest that women who enter late (and were not under medical supervision during organogenesis) probably had poor control. This resulted in malformations due to hyperglycemia or related factors. The results from the diabetic group entering early strongly suggest that other teratogenic mechanisms were present. Regarding early fetal losses, we found that diabetic women in good metabolic control were at no higher risk for spontaneous abortion than control women; the risk of loss increased dramatically as diabetic control worsened; and the overall risk of losing a pregnancy was lower than expected, only 16%. Since these primary analyses were completed, a number of related studies have been completed (see previous reports). A study of infection as a risk factor for early spontaneous abortion has been published. It demonstrates that infection is an uncommon cause of early pregnancy loss. Anti-sperm antibodies have been shown not to be a major factor in first time early pregnancy losses (in contradistinction to recurrent pregnancy loss in later pregnancy). This report has been submitted for publication. Change in insulin requirement during pregnancy is being analyzed.

Project Description:

Dr. James L. Mills, Dr. Jack Lee and Ms. Mary Conley of the DESPR are involved in data editing and analysis. The principal investigators noted on form 6040 also take an active part in data analysis and reporting.

Objectives:

The objectives of the DIEP were to examine the relationship between maternal diabetic control during organogenesis and malformations in the offspring; to identify, if possible, a specific teratogenic factor in diabetic metabolic state; and to compare early fetal loss rates in women with diabetes and non-diabetic control subjects. Secondary goals include investigating the metabolic milieu in early pregnancy, determining risk factors for spontaneous abortion in the non-diabetic population, and relating metabolic events in diabetic pregnancy to complications such as retinopathy in the mother and minor malformations in her offspring. A number of issues relating to metabolic events in diabetic pregnancy, malformations and spontaneous abortion are now being examined.

Methods Employed:

An innovative study design in which women were identified prior to becoming pregnant and monitored closely for early diagnosis of pregnancy was used to address these questions. Women were requested to enroll prior to becoming pregnant and in over 50% of cases subjects monitored basal body temperature in order to identify pregnancy at the earliest possible time. Data were gathered on all pregnancy losses. Malformations were identified at delivery or, when feasible, after abortion. Data from these pregnancies are now being analyzed. Repository specimens collected in anticipation of future need are now being used to answer a variety of questions.

Major Findings:

The major questions have been answered. We found that diabetic women who came into care before the period of organogenesis achieved better results than those who came in later; but their results were still poorer than for non-diabetic control subjects. Differences in maternal glucose levels during organogenesis did not explain the malformations in the offspring of the women who were followed throughout pregnancy. These results suggest that women who enter late (and were not under medical supervision during organogenesis) probably had poor control. This resulted in malformations due to hyperglycemia or related factors. Data from the diabetic group entering early strongly suggest that other teratogenic mechanisms were present. Regarding early fetal losses, we found that diabetic women in good metabolic control were at no higher risk for spontaneous abortion than control women; the risk of loss increased dramatically as diabetic control worsened; and the overall risk of losing a pregnancy was lower than expected, only 16%.

This past fiscal year we published the results of our analysis of the relationship between infection and early pregnancy loss. First trimester infection does not appear to be a risk factor for early spontaneous abortion.

Significance to Biomedical Research and the Program of the Institute:

Our major findings have been that poorly controlled diabetes increases the risk of spontaneous abortion. The risk can be eliminated by good control. Congenital malformations can be reduced by good periconceptional care. However, factors other than glucose are teratogenic and elevated malformation risks are still present in women in moderately good metabolic control.

Anti-sperm antibodies, widely believed to be a significant cause of recurrent, mid trimester abortion, have been shown not to be an important cause of first or second losses in early pregnancy.

Proposed Course of Project:

We are assaying blood specimens collected during pregnancy for micronutrients in order to study their relationship to adverse pregnancy outcomes. We will continue to look at the relationship between ketone levels and bad pregnancy outcomes, and we will examine potential patterns of minor malformations in infants with diabetic mothers. Anti-phospholipid antibodies as a risk factor for spontaneous abortion will be studied. A postulated drop in insulin requirement during the first trimester is being investigated.

Publications:

Chew EY, Mills JL, Metzger BE, Remaley NA, Jovanovic-Peterson L, Knopp RH, Conley MR, Rand L, Simpson JL, Holmes LB, Aarons JH, and the NICHD Diabetes in Early Pregnancy Study. Metabolic control and progression of retinopathy: The Diabetes in Early Pregnancy Study, Diabetes Care 1995;18:631-7.

Simpson JL, Mills JL, Kim H, Holmes LB, Lee J, Metzger B, Knopp R, Jovanovic-Peterson L, Aarons J, Conley M. Infectious processes: An infrequent cause of first trimester spontaneous abortions, Human Reprod 1996;11:668-72.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 0334-13 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Low Birth Weight Across Generations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: M.A. Klebanoff Research Medical Officer EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Office of the Director, DESPR, NICHD (H.W. Berendes); University of Pennsylvania (S. Katz), University of Southern California (B. Mednick)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.25	.25	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The original description of the correlation between birthweights of mothers and their infants was followed by the description of the association between large maternal birth weight and delivery of a macrosomic (>4000 gram) infant. Study of other fetal growth parameters, including length and head circumference, demonstrated that infants of low birth weight mothers were both shorter and lighter than infants of larger mothers, but that the infants were normally proportioned.

In ensuing studies, birth certificates of infants born in Tennessee between 1979 to 1984 were matched with those of their mothers, who were born in Tennessee between 1959 to 1966. Maternal and infant birth weights were again shown to be correlated. In addition, women who were themselves of low birth weight were up to 4 times as likely to have a small-for-gestational age infant as were women who weighed 4000-4499 grams at birth, but low birth weight women were less than twice as likely to have a preterm infant. A group of Swedish women, born from 1955 to 1965, was next studied. Women who themselves were small-for gestational age at birth were at increased risk of giving birth to both small-for-gestational age and preterm infants. Women who were preterm at birth were not at increased risk of either outcome.

Analysis of data from girls who were born in the 1960's as subjects in the Collaborative Perinatal Project and Danish Perinatal Study is currently underway in order to examine their reproductive histories. Small-for-gestational age, preterm and control girls have been located and interviewed. Hospital records of their deliveries have also been retrieved.

Project Description

Objectives:

1) Evaluation of the association between the birth weight of a mother with those of her children.

2) Determination of whether the relationship is due to the presence of outliers, or due to a shifting of the entire birth weight distribution in the second generation.

3) Separation of the second-generation effect of maternal birth weight into independent effects of maternal gestational age at delivery and maternal intrauterine growth.

4) Study of the effects of the perinatal experience of mothers on their subsequent reproductive and health-related outcomes.

Methods Employed:

Work done prior to FY 94 has addressed objectives 1 and 2. Work continuing in FY 94 is directed at addressing objectives 3 and 4. Two studies of similar design are currently being analyzed. In both of these studies, reproductive-age women whose own births had been extensively documented due to their participation in a study of pregnancy were located, and their pregnancy outcomes determined. One study, done under contract by the University of Pennsylvania (subcontracting to Brown University), has traced approximately 120 women who were themselves born preterm, 160 who were small-for-gestational age (SGA), and 350 term, appropriately grown controls. These women were originally subjects in the Philadelphia and Providence cohorts of the Collaborative Perinatal Project. Reproductive histories have been obtained from these women, and medical records from their deliveries have been abstracted. A second contract, awarded to the University of Southern California (subcontracting to the Psykologisk Institut in Copenhagen), has traced approximately 158 parous women who were themselves preterm, 160 who were SGA, 37 who were both preterm and SGA, and 943 parous term controls. These women were originally subjects in the Danish Perinatal Study (1959-61). The extensive record linkage system in Denmark has enabled the collection of data from nearly 100% of all second-generation pregnancies. In addition, the Danish record linkage system has enabled the collection of data on paternal birth weight and (through military draft records) paternal adult height and weight.

Major Findings:

The original description of the correlation between birthweights of mothers and their infants was followed by the description of the association between large maternal birth weight and delivery of a macrosomic (>4000 gram) infant. Study of other fetal growth parameters, including length and head circumference, demonstrated that infants of low birth weight mothers were both shorter and lighter than infants of larger mothers, but that the infants were normally proportioned.

In ensuing studies, birth certificates of infants born in Tennessee between 1979 to 1984 were matched with those of their mothers, who were born in Tennessee between 1959 to 1966. Maternal and infant birth weights were again shown to be correlated. In addition, women who were themselves of low birth weight were up to 4 times as likely to have a small-for-gestational age infant as were women who weighed 4000-4499 grams at birth, but low birth weight women were less than twice as likely to have a preterm infant. A group of Swedish women, born from 1955 to 1965, was next studied. Women who themselves were small-for-gestational age at birth were at increased risk of giving birth to both small-for-gestational age and preterm infants. Women who were preterm at birth were not at increased risk of either outcome.

Work in FY 95 has yielded several findings. In the Danish study, the women had 2046 singleton infants, 24.7% of infants born to SGA mothers were SGA; in comparison, 11.4% of infants born to appropriately-grown mothers were SGA ($p<0.001$). Women who were themselves preterm at birth had a 10.5% risk of giving birth to a preterm infant in any given pregnancy, compared to 6.6% for women born at term ($p=0.04$). SGA mothers were not at significantly increased risk of giving birth to a preterm infant, and preterm mothers were not at significantly increased risk of giving birth to a SGA infant. Adjustment for confounding variables did not change the results. In addition, the association between maternal and infant SGA varied depending on the height of the grandmother. When the grandmother was tall, maternal SGA was not a risk factor for infant SGA. However, when the grandmother was short, maternal SGA added significantly to the infant's risk of being SGA. Additional analyses from this study addressed the antecedents and effects of uncertainty of the gestational age estimate.

Work in FY 96 has continued to expand previous work on maternal factors, and has extended the observations to the study of the association between the father's and infant's birth weights, as well as between father's adult stature and infant birth weight. Compared to fathers who weighed >4 kg at birth, those who weighed 3-3.9 kg had infants who were 109 grams lighter, and those who weighed <3 kg had infants who were 172 grams lighter. This trend was highly statistically significant. Increasing paternal adult height was also associated with increasing infant birth weight ($p=0.03$).

Preliminary analysis of the data from Philadelphia and Providence indicates that the 627 interviewed women had 1131 children. Women who were themselves preterm at birth were not at increased risk of giving birth to a low birth weight infant (relative risk 1.1), but that women who were small-for-gestational age at birth were at significantly increased risk of giving birth to a low birth weight infant (relative risk 1.7, $p<0.05$). Data regarding infant gestational age are currently being edited.

Significance to Biomedical Research and the Program of the Institute:

This project suggests that the pathogenesis of low birth weight begins even before the birth of the mother. This implies that future research on prevention and management of at risk pregnancies should take into account the intrauterine and perinatal experience of a mother. It also implies that changes in the incidence of preterm delivery and intrauterine growth retardation may take several generations to accomplish. Further research is needed to determine the relative contribution of genetic effects versus environmental factors (e.g. smoking) passed across generations on low birth weight.

Proposed Course:

Data analysis is ongoing and two manuscripts are under review.

The Tennessee data were published in the Journal of Pediatrics. The results from Sweden have been published in Pediatrics. A paper describing the Danish study methods was published in Paediatric and Perinatal Epidemiology.

Further results of this project will be published in peer-reviewed journals.

Publications:

Previously listed.

Presentations:

Preterm and small-for-gestational age birth across generations in U.S. whites and blacks. American Public Health Association, 1992.

Uncertain gestational age - a souce of bias in epidemiologic studies. Society for Pediatric Epidemiologic Research, 1995.

SGA and preterm birth across generations. Society for Pediatric Epidemiologic Research, 1995.

Contract Report:

This project has been supported by the University of Pennsylvania, under NICHD contract Number N01-HD-7-2909, and by the University of Southern California, under NICHD contract Number N01-HD-7-2902. These contracts, both entitled "Preterm and Small for Gestational Age Birth Across Generations," were initiated on 3-1-87 and 5-1-87, respectively, and terminated during FY 92. The main objectives of these contracts have been to locate, interview and abstract the obstetrical records of a group of women who were themselves preterm, small for gestational age or normal-sized at birth. The methods employed included interviews with human subjects, and medical record abstraction. During the current fiscal year, interviewing was completed, medical record abstractions have been completed, and data processing and analysis are ongoing. The Project Officer has been extensively involved in the design and execution of these contracts. He designed the sample selection procedures and had primary responsibility for designing the study forms. In addition, he closely monitored the subject location procedures and was responsible for developing solutions to aid in increasing the follow-up rate. He is also responsible for carrying out the data analysis. This contract is essential for locating and interviewing the appropriate research subjects, because such women are otherwise unavailable to NICHD researchers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00361-10 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Child Health Supplement to the 1988 National Health Interview Survey

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD
Other: A. Trumble	Acting Branch Chief	CSB, DESPR, NICHD

COOPERATING UNITS *(if any)*
HLB, CRMC, NICHD (P.C. Scheidt); University of North Carolina at Chapel Hill (J. Kotch); Bar Ilan University (Y. Harel); Centers for Disease Control (D. Jones); Albert Einstein College of Medicine (P. Bijur); NIDCD (H. Hoffman)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.25	.24	.01

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

NICHD sponsored and coordinated the 1988 survey to provide population-based data on children's health. One major collaborative effort analyzed childhood injury risk factors to support effective targeting of interventions since injury is the leading cause of death for children in the U.S. This is the first study to demonstrate that non-fatal injury causes differed considerably from fatal causes, with considerable variation of risk factors by age and socioeconomic characteristics. Analysis of injuries in preschool children by child care patterns showed that use of non-guardian care did not increase risks except for the youngest children of poor, low education mothers who use multiple part-time places of care of all types (center-based, care in their own home or homes of others). Increasing hours in center-based care seemed to lower both total and serious injury risks for children ages 4-5 years. Also, older children of women who are problem drinkers, or of two parents who are both problem drinkers, are at higher injury risk. Another risk factor analysis showed that sports and recreation account for 36% of all medically attended injuries from all causes. Analysis of effects of access to medical care showed that for children without medical care coverage (health insurance or Medicaid), as much as 30% of total injuries and 40% of serious injuries may not have received medical attention compared to children with medical care coverage. The latest studies have shown that children in single adult households are at increased risk regardless of other factors such as poverty or race.

Other projects are focused on factors affecting risks of frequent ear infections, deafness or trouble hearing and related conditions. Effects of maternal smoking on ear infections and respiratory conditions show increased risks.

Project Description

Objectives:

1) To provide estimates of health status for U.S. children including injuries, ear infections, deafness or trouble hearing, and respiratory conditions.

2) To learn the determinants of children's health and injury risks.

Methods Employed:

During 1988 NCHS conducted an interview survey of a representative sample of all households in the U.S. as part of the continuous National Health Interview Survey. About 17,200 sample children were selected. Questions pertained to the child's perinatal and birth period, family structure, child care, health status and behavior. The questionnaire was developed by a cooperative effort from representatives of the NICHD, U.S. Health Resources and Services Administration, National Center for Health Statistics and Child Trends, Inc. The Epidemiology Branch of NICHD had major responsibility for coordination.

Major Findings:

1) No effect on injury risk for preschool children was found for most nonguardian child care use. Risk of serious injury decreased with increasing hours in center-based care at ages 4-5 years. Serious injury risk increased for children <2 years old whose mothers had <12 years of education and who used part-time and multiple places of care across all types of care combined compared to using either full-time or no care.

2) Twelve-month recall of medically attended injuries is affected by severity of the injury, age and sex of the child, and the type of injury event.

3) Non-fatal injuries show a different epidemiological pattern from fatal injuries reflecting interaction of types of exposures and developmental stages.

4) Children of women who are problem drinkers have an elevated injury risk; children with two parents who are problem drinkers are at higher risk.

5) Parents of children without either health insurance or Medicaid are less likely to report medically attended injuries, regardless of injury severity. Studies based on injury reports from treatment sources could be biased when assessing the effects of exposures associated with lack of medical coverage.

6) Sports injuries account for 36% of all medically-attended injuries. Cause and nature of injury are strongly related to age.

7) Children living in single parent households are 40 percent more likely to be injured than those in two-parent households after accounting for race and poverty.

8) Rates of ever having repeated ear infections peak at 31% in ages 3-5 years with significantly higher rates for boys than girls. Low birth weight was

significantly associated with deafness or trouble hearing but not with ear infections.

Significance to Biomedical Research and the Program of the Institute:

This survey documents current health status of U.S. children in relation to accidents, injuries, poisonings, exposure to cigarette smoke and other health conditions. Normative ranges are established and risk factors analyzed. The data enable prioritizing of targeted interventions and research for children through representative U.S. population-based estimates.

Proposed Course:

The article by Overpeck, et al, entitled, "Socioeconomic and racial/ethnic factors affecting nonfatal medically-attended injuries in U.S. children" has been submitted for publication. Injury analyses are almost complete pending final acceptance for publication. Analyses on ear infections, hearing loss and respiratory conditions have either been submitted for publication or are underway.

Publications:

Bijur PE, Trumble AC, Harel Y, Overpeck MD, Jones DH, Scheidt PC. Sports and recreation injuries in U.S. children and youth. Arch Pediatr Adolesc Med 1995;149:1009-16.

Hoffman HJ, Overpeck MD, Hildesheim ME. Factors in the U.S. affecting risk of frequent ear infections, deafness or trouble hearing and related conditions. Proceedings of the Sixth International Symposium on Recent Advances in Otitis Media, Fort Lauderdale, FL, June 4-8, 1995. (In press)

Scheidt PC, Harel Y, Trumble AC, Jones DH, Overpeck MD, Bijur PE. Scheidt and colleagues respond on nonfatal injuries among U.S. children. Letter to the Editor, Am. J. Public Health 1996;86:892.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00369-08 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Adverse Perinatal Events and Subsequent Injury-related Death

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: M.A. Klebanoff Research Medical Officer EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
NIMH (M. Farmer)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 0.05	PROFESSIONAL: 0.05	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Several published studies have indicated that children experiencing asphyxia during the perinatal period are at increased risk of subsequent adolescent suicide. However, these studies used retrospectively ascertained data and were unable to control for the adverse social conditions that often accompany perinatal difficulties.

In the first phase of this project, the names of the approximately 55,000 children who were born during 1959-66 as subjects in the Collaborative Perinatal Project were computerized. During the second phase, the computerized names and other appropriate identifying information were submitted to the National Death Index, and all subjects who have died were identified. In the third phase, the death certificates of these subjects will be obtained and cause of death recorded. Since the Collaborative Project collected extensive data about the subjects' prenatal, perinatal, and childhood histories, it will be possible to study prospectively the relationship between adverse perinatal events and subsequent risk of death, as well as cause of death.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

The purpose of this project is to evaluate the relationship between adverse perinatal events and subsequent rates and causes of death in a population with thoroughly documented prenatal, perinatal and childhood histories.

Methods Employed:

This project will utilize individuals who were born as subjects in the Collaborative Perinatal Project, a multicenter study of pregnancy, delivery and childhood that prospectively enrolled approximately 60,000 pregnancies from 1959-66. The subjects' names, which do not exist on computer files, were entered onto a computer. This task was completed in the winter of 1993. Following this, the names, along with other required identifying information, were submitted to the National Death Index, where it was possible to match them to all deaths in the United States from 1979 to the present. Results were obtained in the spring of 1994. For subjects who have died, the death certificate will be obtained and cause of death coded. Rates and causes of death will be compared to determine the effect of perinatal asphyxia on subsequent adolescent and young adult mortality.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute:

This project will provide valuable additional data on the relationship between perinatal events and subsequent young adult death, and will enable the intervening role of adverse social situation to be investigated. The project will add significantly to the present state of knowledge about the long term effects of perinatal events.

Proposed Course:

The preparation of the data tape has been completed. The tape was submitted to the National Death Index in the winter of 1993, and results obtained in the spring of 1994. Death certificates of possible matches will be obtained using FY 97 funds.

Publications: None yet

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00373-08 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Calcium Supplementation in Pregnancy for the Prevention of Preeclampsia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.J. Levine	Research Medical Officer	EB, DESPR, NICHD
Others:	R. DerSimonian	Mathematical Statistician	BMSB, DESPR, NICHD
	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
	M.A. Klebanoff	Research Medical Officer	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Univ. of Alabama (JC Hauth, G Hortin) Case Western Reserve Univ. (PM Catalano); Univ. of New Mexico (LB Curet); Oregon Health Sci. Univ. (C Morris); Univ. of Tennessee (BM Sibai); The Emmes Corp. (J Esterlitz); Biomedical Res. Inc. (J Leaf)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The results of ten clinical trials suggest that supplemental calcium may prevent preeclampsia. Methodologic problems, however, and differences in study design limit the credibility of the results and their generalizability to other patient populations. Moreover, none of the trials has reported the outcome of systematic surveillance for urolithiasis, an important possible complication of treatment. In response to the need for a definitive evaluation of the effects of calcium supplementation, NICHD has conducted a trial at five U.S. university medical centers. Healthy nulliparous patients were randomly assigned to receive either 2 g ingested supplemental calcium daily (n=2250) or placebo (n=2250) in a double-blind study. Medication was administered beginning between 13 and 21 completed weeks of gestation and continued until the termination of pregnancy. Monitoring for the major study endpoints - pregnancy-associated hypertension and proteinuria, preeclampsia, eclampsia, and HELLP syndrome - and for urolithiasis was systematic, standardized, and thorough. It included measurement of blood pressure, proteinuria, and hematuria at uniformly scheduled prenatal clinic visits and surveillance for hypertension and proteinuria during labor, delivery, and the first 24 hours postpartum. CPEP has adequate statistical power to detect a reduction of 43 percent in preeclampsia risk in the calcium group.

Project Description

Objectives:

The purpose of this investigation was to evaluate the efficacy of 2 gm/day ingested supplemental calcium for reducing the incidence of important hypertensive disorders of pregnancy - preeclampsia, eclampsia, and HELLP syndrome - in healthy nulliparous women.

Methods Employed:

The study was a multicenter, randomized, double-blind, placebo-controlled clinical trial. Besides nulliparity, study subjects lacked known risk factors for preeclampsia. Blood pressure was monitored at prenatal clinic visits by trained nurses using mercury sphygmomanometers and cuffs of appropriate size. Proteinuria was measured by total protein excretion in 24-hour urine collections and in random urine specimens, by dipsticks and by protein/creatinine ratios.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute:

Should calcium supplementation be proven beneficial, it may become a part of routine prenatal care for the purpose of reducing the risk of hypertensive disorders of pregnancy and decreasing associated maternal and perinatal morbidity and mortality.

Proposed Course:

The trial has been completed. Analysis of the data is underway. A manuscript summarizing the major outcomes will be submitted for publication during FY 96. During FY 97 several related manuscripts will be prepared for publication, and laboratory analysis of urine specimens obtained from study subjects will be completed.

Publications:

Levine RJ, Esterlitz JR, Raymond EG, DerSimonian R, et al. The trial of calcium for preeclampsia prevention (CPEP): rationale, design, and methods, Controlled Clin Trials 1996 (in press).

Levine RJ, Raymond E, DerSimonian R, Clemens JD. Preeclampsia prevention with calcium supplementation, Clin Appl Nutr 1992;2:30-38.

Contract Report:

The project has been supported by The Emmes Corporation, the University of Alabama, Case Western Reserve University, the University of New Mexico, Oregon Health Sciences University, the University of Tennessee, and Biomedical Research Incorporated under the following respective contract numbers: N01-HD-1-3121, N01-HD-1-3122, and N01-HD5-3246, N01-HD-1-3125, N01-HD-1-3124, N01-HD-1-3123, N01-HD-1-3126, and N01-HD-2-3154. During FY 96 $167,981 has been allotted to these contracts. The major objective of the contracts is to provide support to five clinical centers, for enrollment and follow of patients for the trial; to a data coordinating center, responsible for all data management and analysis; and to a central repository for storage of laboratory specimens obtained from study subjects; and to a university laboratory for analysis of urine specimens. The trial was completed during the current fiscal year. Laboratory analysis of urine specimens obtained from study subjects, analysis of the data, and the preparation of manuscripts for publication are currently underway.

The above contracts are essential since NICHD does not have facilities for enrolling and monitoring pregnant women, sufficient statistical staff to maintain a data coordinating center, or the facilities and personnel necessary for analyzing laboratory specimens or for operating an ultra-low temperature storage facility.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00379-07 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Data Analysis from the Vaginal Infections and Prematurity Study

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M.A. Klebanoff	Research Medical Officer	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*

PAMA, CRMC, NICHD (R. Nugent); NIAID (M.F. Cotch); Research Triangle Institute (A.V. Rao); Columbia University (J. Regan); University of Alabama, Birmingham (R. Goldenberg)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.25	.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Vaginal Infections and Prematurity (VIP) Study, jointly sponsored by NICHD and NIAID, is a prospective study designed to investigate the relationship between genital tract colonization with various microorganisms and the subsequent development of preterm birth. It also incorporates a clinical trial of erythromycin to prevent preterm birth among women colonized with *Ureaplasma urealyticum*, *Chlamydia trachomatis*, and group B streptococcus. In addition, the VIP collected a wealth of additional data on a variety of factors possibly linked to pregnancy outcome. This project will analyze these factors.

Analyses undertaken to date include the predictive value of vaginal Gram stain in the identification of group B streptococcus, the descriptive epidemiology of group B streptococcal carriage, the relationship between reported physical activity and preterm birth, the effect of treatment with erythromycin on pregnancy outcome among women colonized with group B streptococci, and the association between sexual intercourse during pregnancy preterm birth among colonized with different genital microorganisms, effect of group B streptococcal colonization on pregnancy outcome, the association between bacterial vaginosis and pregnancy outcome, the effect of treatment with erythromycin on pregnancy outcome among women colonized with *Chlamydia trachomatis*, and ethnic differences in genital flora.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The objective of this project is to investigate factors associated with adverse pregnancy outcome in the Vaginal Infections and Prematurity (VIP) data set.

Methods Employed:

This study, sponsored by NICHD and NIAID, is a prospective study designed to investigate the relationship between genital tract colonization with various microorganisms and the subsequent development of preterm birth. It also incorporates a clinical trial of erythromycin to prevent preterm birth among women colonized with *Ureaplasma urealyticum*, *Chlamydia trachomatis*, and group B streptococcus (GBS). In addition, the VIP collected a wealth of additional data on a variety of factors possibly linked to pregnancy outcome.

Major Findings:

Several analyses have been conducted to date. The utility of a cervicovaginal Gram stain as a screening tool for carriage of group B streptococcus was evaluated. The sensitivity, specificity, positive and negative 1predictive values were 28, 69, 17, and 81% at mid gestation, and 34, 72, 18, and 86% at delivery. The positive and negative predictive values differed little from the baseline prevalence of the organism. It was concluded that the Gram stain was not a useful screening tool for the detection of group B streptococcal carriage.

An additional analysis detailed the descriptive epidemiology of group B streptococcal colonization during pregnancy. Various factors associated with group B streptococcal carriage were described. For example, group B streptococcus was more common among older women and women of lower parity; it was less common among women currently living with their partner, and less common among current smokers. Increasing years of sexual experience resulted in a decreased risk of colonization. Group B streptococcus was present more frequently among women colonized with *Candida* species, but group B streptococcus was not more common among women colonized with *Chlamydia*, *Ureaplasma* or *Trichomonas*, compared to women not colonized with these organisms.

The analysis of the relationship between group B streptococcal carriage and pregnancy outcome has concluded. Preliminary analyses did not demonstrate a significant association between carriage of the organism and either preterm delivery or low birth weight. Similarly, these adverse outcomes were not more common among women heavily colonized with group B streptococcus than among those lightly colonized. However, further analysis demonstrated that nearly 25% of women received a variety of antibiotics for clinical indications during the third trimester. Among women who did not receive clinically indicated antibiotics, increasing density of streptococcal colonization was associated with a significantly increasing risk of delivery before 32 weeks, as well as with a significantly increasing risk of giving birth to a low birth weight (<2500 gram) or very low birth weight (<1500 gram) infant. Results from the clinical trial of erythromycin indicate that treatment of streptococcal carriers with erythromycin does not result in a reduced occurrence of early delivery or delivery of a low birth weight infant.

A fourth analysis investigated the relationship between reported physical activity and preterm birth. Prolonged periods of standing were modestly associated with preterm birth (odds ratio for ≥8 hours/day of standing = 1.31). Heavy work/exercise was not associated with preterm birth (odds ratio for ≥4 hours/day of heavy work = 1.04). The proportion of infants born preterm did not differ among women employed in predominantly standing, active or sedentary occupations. Physical activity was not associated with gestational age-adjusted birth weight. In an updated analysis, prolonged standing was no longer associated with preterm birth.

An additional analysis evaluated the association of reported frequency of sexual intercourse at 23-26 weeks and preterm birth. Among all women, sexual intercourse was associated with a 20% reduction of preterm birth. However, the effect of sexual intercourse was dependent on the presence of certain microorganisms. Among women who were not colonized with *Trichomonas vaginalis*, sexual intercourse was associated with a reduced risk of preterm birth, but among women who were colonized with this organism, intercourse was not associated with preterm birth. Similar results were obtained for colonization with *Mycoplasma hominis*. Conversely, *T. vaginalis* and *M. hominis* were risk factors for preterm birth only among women reporting frequent sexual intercourse.

In another analysis, the association between the presence of bacterial vaginosis and pregnancy outcome was studied. Bacterial vaginosis was present in 16% of the study women, and was associated with the delivery of a preterm-low birth weight infant (odds ratio 1.4, 95% confidence interval 1.1-1.8).

A further analysis evaluates the different vaginal flora among women of different ethnic groups. It was found that most of the pathogenic organisms were found most commonly among black women, followed by Hispanic, white and Asian women. In particular, bacterial vaginosis was three times as prevalent among black compared to white women and Trichomonas was five times as prevalent among black women. Ongoing analysis is addressing the difference in risk factors for bacterial vaginosis among black, white, Hispanic and Asian women. While this condition shares a few of the typical characteristics of a sexually transmitted disease in white women, there were no risk factors for this condition among blacks. This is, it appears to be almost randomly distributed among black women.

Significance to Biomedical Research and the Program of the Institute:

Preliminary results from the clinical trial indicate that treatment of all women colonized with group B streptococcus is unlikely to reduce their risk of preterm or low birth weight delivery. It is possible, however, that treatment of the more densely colonized women might have some benefit. These analyses indicate that Gram stain is not an effective tool to screen for group B streptococcal carriage, implying that other screening methods for this important perinatal pathogen need to be devised. The results of the analysis of the relationship between activity and preterm birth have both clinical and research implications. Practical implications are that for most women with uncomplicated pregnancies, there is no need to restrict activity in the hopes of preventing preterm birth.

Important research implications are that on a population basis, changes in maternal activity during pregnancy are not likely to have large effects on the rate of preterm birth, and that unmeasured differences in socioeconomic status between women reporting different amounts and types of physical activity were likely to have accounted for previous positive results on this subject.

The results of the analysis of sexual intercourse shed important new light on the role of infection in the pathogenesis of preterm birth. First, they imply that for most pregnant women, intercourse does not adversely affect the pregnancy. Second, the results suggest that when certain organisms are present in the vagina, their introduction into the cervix (as might happen during intercourse) can lead to preterm labor.

The analyses of bacterial vaginosis have identified an important, treatable risk factor for preterm birth.

Proposed Course:

The analysis of chlamydial colonization on pregnancy outcome is continuing.

Publications:

Goldenberg RL, Klebanoff MA, Nugent RP, Krohn MA, Hillier SL, Andrews WW. Bacterial colonization of the vagina in four ethnic groups, Am J Obstet Gynecol 1996;174:1618-21.

Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, Edelman R, Cotch MF, Pastorek J, Rao AV, McNellis D, Regan JA, Carey JC, Klebanoff MA. The association of bacterial vaginosis, bacteroides, and *Mycoplasma hominis* with preterm, low birth weight delivery, N Engl J Med 1995;333:1737-42.

Regan JA, Klebanoff MA, Nugent RP, Blackwelder WC, Eschenbach DA, Lou Y, et al. Colonization with group B streptococci in pregnancy and adverse outcome, Am J Obstet Gynecol 1996;174:1354-60.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00383-06 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analyses of Data from the Collaborative Perinatal Project

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M.A. Klebanoff Research Medical Officer EB, DESPR, NICHD

COOPERATING UNITS *(if any)*

NEI (E. Chew); University of Maryland (D. Nagey); Packard Foundation (P. Shiono); McGill University (M. Kramer)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: .05	PROFESSIONAL: .05	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Collaborative Perinatal Project (CPP) represents a rich data set for the investigation of a variety of topics related to maternal and child health.

Currently active areas of investigation include the descriptive epidemiology of congenital cataract, the association between maternal smoking and childhood cancer, and the association between elevated maternal serum levels of alpha fetoprotein at 14-20 weeks and subsequent stillbirth.

Project Description

Objectives:

1. To determine the association between neonatal hyperbilirubinemia and neurodevelopmental abnormalities at age 7-8.

2. To state the descriptive epidemiology of strabismus and congenital cataract.

3. To determine the association of elevated maternal serum alpha fetoprotein at 14-20 weeks and subsequent stillbirth.

4. To determine the recurrence risk of RDS in preterm siblings.

5. To determine whether perinatal exposure to vitamin K is associated with an increased risk of cancer during childhood.

Methods Employed:

The Collaborative Perinatal Project (CPP) enrolled pregnant women entering prenatal care at 12 university hospitals across the United States from 1959 to 1966. The women were followed throughout pregnancy, labor and delivery, and their children received multiple physical, neurological and psychological exams during their first 7 to 8 years of life. Maternal serum was collected at multiple times during pregnancy. Extensive data were collected on a wide variety of obstetric and pediatric conditions.

One investigation using this dataset completed during FY94, in collaboration with Dr. Thomas Newman of the University of California at San Francisco, evaluated the association between neonatal hyperbilirubinemia and neurodevelopmental outcome at age 7-8. The CPP required that serum bilirubin be measured on every neonate according to a fixed protocol. Extensive psychologic testing was performed at age 7, as was a detailed neurologic examination. In addition, a smaller group of children underwent tests of speech and hearing function at age 8. This analysis compared the Wechsler IQ at age 7 of children with different values of peak neonatal serum bilirubin. In addition, the presence of an abnormal neurologic examination at age 7 was studied among children with different peak bilirubin values. Finally, hyperbilirubinemic and non-hyperbilirubinemic children were compared for the presence of sensorineural hearing loss at age 8. The benefits of treating hyperbilirubinemia in otherwise healthy term infants are controversial. Since the clinical management of healthy term infants has changed little over the past decades, data of this age are still relevant. In fact, the absence of phototherapy as a treatment for hyperbilirubinemia makes these data even more valuable, as it would be very difficult to assemble a cohort of neonates with untreated hyperbilirubinemia today.

A second investigation, done in collaboration with personnel at the Epidemiology Branch of the National Eye Institute, is addressing the descriptive epidemiology of strabismus and congenital cataract. This condition is relatively common, affecting 2-3% of all children. Review of the literature reveals few studies describing risk factors for strabismus. The CPP conducted detailed neurologic examinations at ages 1 and 7, making it well-suited for the study of this condition.

A third investigation, done in collaboration with Dr. David Nagey of the University of Maryland, evaluated the predictive value of elevated maternal serum alpha fetoprotein for stillbirth in otherwise normal infants. In this study alpha fetoprotein levels in serum drawn at 14-20 weeks gestation from mothers delivering non-malformed stillborn infants were compared to levels in women delivering liveborn infants. Each stillborn case was matched to 4 liveborn controls for study site, race, gestation at which blood was obtained (±3 days), and years elapsed since the serum was drawn (±2 years). The fraction of cases and controls with alpha fetoprotein levels of greater than 3 multiples of the median was compared.

A fourth investigation studied the risk of childhood cancer following perinatal vitamin K exposure. Two previous reports have indicated that children who received this drug during the neonatal period are at approximately twofold increased risk of developing cancer. Since nearly every child born in the United States during the past several decades has received intramuscular vitamin K, up to half of all childhood cancer might be attributable to the drug. The CPP is an ideal cohort in which to study this issue, as it spanned the era when vitamin K exposure changed from being rare to being common.

A fifth investigation studied the association between maternal smoking during pregnancy and cancer during childhood. In this cohort, approximately half of the women smoked during pregnancy, so the power to detect associations would be maximized.

A sixth investigation, in collaboration with researchers at McGill University, studied the recurrence of respiratory distress syndrome in successive preterm siblings.

Major Findings:

Peak serum bilirubin was not associated with 7-year IQ in either black or white infants. Among white infants the IQ of children whose bilirubin value was ≥20 mg/dl was 105.0, compared to 103.4 for children whose bilirubin value was <20 mg/dl. The corresponding values for black children were 91.0 and 93.3. Definitely abnormal neurologic examinations at age 7 were not more common among children whose peak neonatal bilirubin value was ≥20 mg/dl (4.5%) compared to those who were <20mg/dl (3.8%). However, the frequency of abnormal or suspicious neurologic examinations increased in a stepwise manner from 14.9% in children with bilirubin values <10 mg/dl to 22.4% in those with peak bilirubin values of ≥20 mg/dl (p<0.001). This increase was accounted for by minor, non-specific motor abnormalities.

In the alpha-fetoprotein analysis, the odds ratio for fetal death in pregnancies with serum alpha-fetoprotein values >3.0 multiples of the median was 1.98 (p=0.018). However, the sensitivity of elevated alpha fetoprotein for prediction of fetal death was only 8.5%; the specificity was 95.7%. In a modern-day population with a probability of late fetal death of 1%, the predictive value of a positive test would be only 2%, which is not sufficiently high to be of clinical utility.

There were 48 children in the CPP who developed cancer after the first day of life, including 16 who developed leukemia. This resulted in a cumulative incidence of cancer and leukemia of 1.1 and 0.4 per 1000, respectively, by age 7-1/2. Neonatal exposure to vitamin K was found not to be associated with the development of cancer during childhood (odds ratio 0.84, 95% confidence interval 0.41 to 1.71), or of leukemia (odds ratio 0.47, 95% confidence interval 0.14 to 1.55).

The results of the strabismus analyses indicate that 3% of the cohort developed esotropia and 1.2% developed exotropia. Esotropia was approximately twice as common in white as in black children, but exotropia did not differ by race. Both types of strabismus were more common in low birth weight infants. Maternal cigarette smoking during pregnancy was associated with an increased risk of both types of strabismus; this finding was independent of the birthweight-reducing effect of smoking.

There were 51 children with cancer, for a cumulative incidence of cancer of 1.1 per 1000 by 96 months of age. This is 3 more than the vitamin K analysis, because it includes cases diagnosed on the first day of life. Maternal smoking was determined at each prenatal visit; 52% of mothers reported smoking at one or more visits. By age eight, cancer occurred in 1.4 per 1000 children whose mothers did not smoke during pregnancy, compared to 0.9 per 1000 children whose mothers smoked (p = 0.15 by logrank test); the hazard ratio was 0.67 (95% CI 0.38-1.17). This indicates that smoking is not associated with an increased risk of cancer during childhood.

Significance to Biomedical Research and the Program of the Institute:

Each of these analyses involves a problem of relevance to child health. Treatment of jaundice results in large health care expenditure for the United States, due to the frequent extension of hospital stays for otherwise normal newborns. Furthermore, the need for this treatment is controversial. If it were to be shown that physiologic hyperbilirubinemia is not harmful to normal term infants, management of these infants might require reevaluation. Study of risk factors for strabismus will further our understanding of this common condition. The study of serum alpha fetoprotein will demonstrate the stability of this compound in long-term storage. It will also determine whether or not this test is a clinically useful marker for predicting stillbirth. The data on vitamin K provide important reassurance regarding the safety of this commonly used drug.

Preliminary results of the cataract analysis revealed few risk factors for the condition - other than low birth weight. Ongoing analysis will evaluate the association between cataract and other congenital anomalies.

Proposed Course:

Data analyses of the cataract and alpha-fetoprotein results is continuing, and results will be submitted to peer-reviewed journals. The report of the association between maternal smoking and childhood cancer is under review.

Publications:

Nagourney BA, Kramer MS, Klebanoff MA, Usher RH. Recurrent respiratory distress syndrome in successive preterm pregnancies: Are some women "RDS-prone?" J Pediatr, in press.

Presentations:

Maternal Smoking During Pregnancy and Childhood Cancer. Society for Pediatric Research, 1995.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00384-04 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Field Trial of Oral Cholera Vaccines in Bangladesh

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Johns Hopkins School of Public Health (D.Sack); International Center for Diarrheal Disease Research, Bangladesh

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00385-04 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Epidemiology of Rotavirus Infections in Bangladesh

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS (if any)
James Gamble Institute for Medical Research, Cincinnati, OH (R. Ward)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)
This project has been terminated.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00386-05 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Evaluation of a Water-Sanitation Intervention in Egypt

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
UNICEF, Egypt

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this field project is to evaluate the impact of a combined water-sanitation intervention on the occurrence of pediatric diarrhea and other targeted outcomes in a study population residing in the Assiut governorate of Upper Egypt.

20 villages, which together included a population of approximately 10,000 persons, were randomly assigned to receive the intervention (N=10) or no intervention (N=10). The intervention consisted of provision of India Mark II tubewells and household latrines, together with an educational package stressing personal hygiene and water behaviors. To assess the impact of the intervention, all families in these villages were followed with logitudinal surveillance during a one-year follow-up period.

Although the baseline incidence of diarrheal episodes (per 1000 person-days of follow-up) in children under three years of age was similar in intervention (29) and control (29) communities, after zero-time rates of diarrhea decreased 17% (p<.0001) in the intervention relative to the control communities. Protective associations were slightly higher for bloody diarrhea (25%, p<.05) and persistent diarrhea (26%, p<.0001), suggesting that the intervention might have reduced not only the risk of diarrhea but also the severity of diarrhea. In contrast, no impact on the occurrence of respiratory infections was evident, indicating that the decline of diarrhea in the intervention communities was not likely to be due to ascertainment bias. It was of particular interest that intervention communities exhibited a marked increase of per capita water consumption, but that the new pumps continued to produce water that was contaminated by fecal coliforms. There data suggest that the salutory effect of the intervention may have been mediated, in part, by increased water consumption, though the water itself was fecally contaminated. Analyses are now continuing.

Project Description:

Personnel: Dr. John Clemens of the Epidemiology Branch, DESPR, directs this study. Subjects were collected under contract by UNICEF to SPAAC, a private research firm in Cairo. Mr. Malla Rao of the DESPR is conducting the analysis.

Objectives:

1. To assess whether an intervention intended to provide tubewells and family latrines, as well as water-hygiene education, can improve salutary water-sanitation behaviors, as well as other measures of sanitation, such as environmental cleanliness, microbiological purity of stored water, and overall water usage.

2. To assess whether the intervention also results in a diminution in the rate of pediatric diarrhea in targeted villages.

Methods Employed: After assembly of consenting families and acquisition of informed consent, participating villages were randomly allocated, using stratified randomization, to intervention an control groups. Following implementation of the intervention, all participating families were visited on a weekly basis, and, at varying intervals, measurements of target outcomes were made with use of direct questionnaires, observations of relevant behaviors in the home environments, and observations of water use at the pumps, microbiological sampling of water in the homes and at the pumps.

Major Findings: The major finding obtained during the fiscal year was that children in the intervention villages experienced a 17% reduction in the rate of diarrhea, an effect that applied to both watery and non-watery diarrhea, and to acute and persistent diarrhea. Importantly, no protection was observed against acute respiratory infections, as would be predicted on epidemiological grounds. Moreover, the prevention of diarrhea associated with the intervention occurred despite increased per capita consumption of fecally contaminated water in the intervention communities.

Significance to Biomedical Research and the Program of the Institute: Pediatric diarrhea constitutes a major source of morbidity and mortality worldwide. Efforts to control pediatric diarrhea have, in the past, focused largely on improved case management, largely through promotion of oral rehydration therapy. However, these efforts have done relatively little to control the problems of dysentery and persistent diarrhea, for which preventive measures may be necessary. This evaluation of a pragmatic intervention which is already being incorporated into public health strategies by UNICEF, demonstrates that a multipronged water-sanitation program may indeed be capable of complementing the salutary effects of the case-management strategy.

Proposed Course: During the coming fiscal year, final analyses of the impact upon both proximate (e.g., behavioral, environmental) and ultimate outcomes (pediatric diarrhea and nutritional status) will be conducted. The project will also provide a rich set of data to address several issues in diarrheal disease epidemiology, such as the impact of breast feeding on the risk of diarrhea, and the interaction of this association with environmental cleanliness; and the relationship between water use and water cleanliness with risk of pediatric diarrhea.

Publications: Wassif N, Loza S, Rao M, Terreri N, Clemens J. Impact of a water-sanitation program in remote villages of Upper Egypt: Results of a large-scale community experiment. Abstract. US-Japan Cooperative Science Meeting. Kiawah, October, 1995.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00389-04 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies of the Epidemiology of Pediatric Shigellosis in Bangladesh

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.D. Clemens	Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
International Center for Diarrheal Disease Research, Bangladesh; Johns Hopkins University (F. Ahmed)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.20	.20	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00392-05 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Fatal Injuries to U.S. Infants, 1983-1987

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.A. Brenner	Staff Fellow	EB, DESPR, NICHD
Other:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD
	H.W. Berendes	Director	DESPR, NICHD
	A.C. Trumble	Computer Specialist	CSB, DESPR, NICHD
	R. DerSimonian	Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The National Center for Health Statistics linked birth and death records for U.S. infants provides the first opportunity to assess the demographic, socioeconomic and prenatal risk factors associated with deaths due to injuries in infants less than one year of age. U.S. studies of childhood deaths have been limited to the extremely sparse information available on standard death certificates. Both descriptive and risk factor analysis of infant deaths for 1983-1991 are being prepared for publication. Deaths due to homicide, the leading cause of injury death in infancy, are being examined separately to identify infants to be targeted for special interventions to prevent these deaths.

Of particular interest is a recent methodological analysis of infant injury deaths that are classified as "undetermined intent." By evaluating risk factor profiles of such injuries, it may be possible to classify these injuries as likely intentional or likely unintentional. This study has enormous public health implications, as such injuries could increase death rates from homicide in the less than one year age group by 10-15% in the U.S.

Project Description

Objectives:

1) To examine national fatal injury patterns among infants according to previously unknown risk factors, such as maternal education.

2) To determine the degree of bias introduced by potential misclassification of injuries of undetermined intent.

3) To identify characteristics of infants dying from homicide that would enable targeting of preventive interventions towards those at highest risk.

Methods Employed:

Data for this study were provided from the National Center for Health Statistics linked birth/infant death data sets for the years 1983 through 1991. National, cause specific, injury death rates among infants less than one year of age were calculated. Univariate and multivariate analysis examined the effect of several maternal sociodemographic variables (e.g. maternal education) and perinatal variables (e.g. birth weight) on injury outcome.

Major Findings:

The linked file data for the 1983-1991 national birth cohorts were pooled and analyzed. There were 10,702 deaths due to injury over this nine year period, resulting in a death rate of 31/100,000 live births. Twenty two percent of deaths were classified as homicides, 74% as unintentional and 4% as undetermined intent. Potential risk factors ascertained from birth certificate information included both maternal and infant factors. Univariate analysis showed a significant association between each variable examined and injury death. Maternal factors, such as education, maternal age and prenatal care were more strongly associated with injury death than were "infant" characteristics such as gender and birthweight. Many of the characteristics were highly correlated, such as maternal age and marital status.

For all variables examined, the population profile for injury deaths of undetermined intent resembled the profile for intentional injuries more strongly than that for unintentional injuries. For most of the maternal factors, relative risks for deaths classified as undetermined intent were the same or higher than the RR's for deaths classified as intentional. For example, the RR of death for infants born to mothers who were <15 years of age as compared to those ≥25 years was 6.8 for intentional injuries, 6.9 for injuries of undetermined intent and 3.8 for unintentional injuries. Deaths of undetermined intent are apparently both statistically and logically combined with deaths due to intentional injuries.

Homicides were the leading cause of infant injury death with 2,276 deaths from 1983-1991. More than half of the infants were killed before the end of their fourth month of life; 25% by the end of the second month. Highest risk factors were maternal age <17 years, late or no prenatal care, and having a second or third child by age 19. The latter are at 12 times greater risk compared to children born to mothers ≥25 years who have two or more children. Interventions targeting very young pregnant girls before birth of the infant would be most effective.

Significance to Biomedical Research and the Program of the Institute:

Injuries are the third leading cause of postneonatal death yet relatively little is known about the risk factors for injury death in this age group. The linked birth/infant death data set gives us a unique opportunity to examine risk factors for injury death in this age group. These results, which identify several maternal factors which put infants at particularly high risk of injury death should help public health workers in targeting preventive interventions towards the families of those infants who are likely to be at greatest risk. In addition, preliminary results from the methodological analyses of injuries of undetermined intent indicate that these injuries are more likely intentional than unintentional in nature. These findings suggest that fatality rates from intentional injuries could be 10-15% greater than previously recognized.

Proposed Course:

Initial descriptive findings were reported at the joint Society for Pediatric Research/American Pediatric Society/Ambulatory Pediatric Association meetings in 1993. Findings of the methodologic study on the classification of injuries of undetermined intent were presented at the meeting of the American Public Health Association in October of 1995 and findings from the study examining infant homicide deaths were presented at the 3rd International Conference on Injury Prevention and Control in February of 1996. Several manuscripts, both descriptive and analytic, are currently being prepared.

Publications: None at this time.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00801-21 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies Based on the Medical Birth Registries of Norway and Sweden

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A.A. Herman	Visiting Scientist	EB, DESPR, NICHD
Others:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
	J.F. Troendle	Staff Fellow	BMSB, DESPR, NICHD
	A. Lindley	IRTA Fellow	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
NIDCD (H. Hoffman); Univ. of Bergen, Norway (P. Bergsjo, L. Irgens); Univ. of Trondheim and Natl. Inst. of Publ. Hlth., Oslo, Norway (L. Bakketeig, A. Arntzen)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies have focused on: 1) the impact of smoking on head circumference and intrauterine growth - especially head sparing, 2) size at birth as measured by head circumference, crown-heel length, and birth weight in relation to gestational age and perinatal mortality, 3) the tendency to repeat similar birth weight and gestational age in subsequent pregnancy outcomes to the same mothers, 4) perinatal mortality in relation to order of birth and size of sibship, 5) epidemiologic risk factors for preterm birth, 6) epidemiologic risk factors for small-for-gestational age births, 7) contribution of multiple births to perinatal mortality rates.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

These studies have been based on unique perinatal data sets, the Medical Birth Registries of Norway and Sweden. The objective is to compare United States data with that of other national population-based data sets from Scandinavia. These comparisons include birth weight-for-gestational age percentiles, birth weight-specific perinatal mortality rates and a variety of other sociodemographic, lifestyle, and health care comparisons.

Methods Employed:

Analytic methods include indirect standardization of mortality rates by birth weight and gestational age, life-table analyses, discriminant analysis, examination of bivariate distributions using contour diagrams, fuzzy clustering of growth retarded and non growth retarded infants, non-parametric logistic regression, and other procedures applicable to population-based data. The epidemiologic approach is that of a "prospective" cohort design, which permits analyses by case-control, cross-sectional or longitudinal means.

Major Findings:

At low birth weight the variance of last menstrual period based gestational age is wide and the distribution is positively skewed toward higher values. In one study the variance of gestational age decreases rapidly as birth weight increases, skewness decreases and kurtosis increases in approaching the mean of the birth weight distribution. Some of the wider variance and positive skewness of gestational age at low birth weight appears to reflect heterogeneity of intrauterine growth, in which infants with high values of gestational age are growth retarded. We show by partitioning each birth weight group into two groups of infants with different gestational age distributions, that at low birth weight, infants with low gestational ages have higher neonatal mortality rates but lower fetal mortality rates than infants with a higher gestational age for birth weight. The differences in mortality described between small infants at different gestational ages suggest that infants with a high LMP-based gestational age have experienced a slower rate of intrauterine growth. Some authors interpret the distributional characteristics as indications of systematic error in last menstrual period based assessment of gestational age. It appears from this study that the extent of systematic error in the estimation of LMP based gestational age may have been overstated in the past. The fuzzy clustering approach used to classify infants into IUGR groups allowed us to bypass the categorical approaches of IUGR classification which are prone to misclassification. Analyses of Scandinavian and United States data showed that among preterm infants African-American excess infant mortality increases from twice to sixteen times that of whites as birth weight and gestational age increases. Among growth retarded infants the excess mortality increases from twice to eight times as birth weight and gestational age decreases.

Significance to Biomedical Research and the Program of the Institute:

The studies using this rich data source have provided information on several issues pertinent to programs of the Institute. The "repeater" studies have helped to elucidate some of the etiologic problems of prematurity and low birth weight. Studies of fetal and infant mortality, especially comparisons with available United States data sets, will aid understanding of the cause-specific mortality categories in which the United States rates may be higher than Scandinavian rates. The studies of IUGR classification allow for more complete examination of mortality and morbidity risk associated with growth abnormalities. The analysis of birth weight and gestational age specific mortality points to deficits in the care of preterm an growth retarded African-American infants.

Proposed Course:

Numerous research publications have resulted from these collaborative efforts on the Swedish and Norwegian Medical Birth Registries. Further collaborations using data from both of these registries are continuing with Dr. Per Bergsjo, Professor, Department of Obstetrics and Gynecology, University of Bergen, Norway. Dr. Allen Herman continues the analysis of perinatal mortality risks in relation to various size at birth measurements using fuzzy clustering and nonparametric logistic regression techniques.

Publications: None at present.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00861-14 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Assessment of In-Utero Fetal Growth Patterns in Relation to Outcome at Birth

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A.A. Herman	Visiting Scientist	EB, DESPR, NICHD
Other:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS *(if any)*
NIDCD (H. Hoffman); U. Trondheim, Norway (G. Jacobsen, L. Bakketeig, K. Sommerfeldt); U. Bergen, Norway (P. Bergsjo, T. Markestad); U. Uppsala, Sweden (G. Lindmark, G. Ahlstein).

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This is a prospective study of risk factors associated with intrauterine growth retardation. Recruited were pregnant women before 17 weeks gestation at the University of Alabama in Birmingham and University of Trondheim, Norway (in collaboration with the Universities of Bergen and Uppsala) for the period of January 1986 through June 1988. The children are followed for five years to monitor cognitive, physical and behavioral development.

Analysis will focus on:
- The intrauterine growth curve.
- The impact of socioeconomic factors, psychosocial factors, behavioral risk factors, nutritional factors on intrauterine growth.
- Cognitive, behavioral, psychological and nutritional status at 5 years among group with different rates of intrauterine growth, and different postnatal social and environmental factors.
- Postnatal growth for growth retarded infants.

Project Description:

Objectives:

These studies will evaluate the predictive value of several obstetrical tests, including diagnostic ultrasound measurements, in assessing fetal growth in-utero and in relation to outcome at delivery. Follow-up examinations of infants will evaluate morbidity status and developmental progress, including several growth parameters, at one and five years of age.

Methods Employed:

This is a population-based study conducted in Alabama and Scandinavia. The population in Alabama focuses on the population of Jefferson County, which includes Birmingham. The health care for these women is provided for by the county health department in affiliation with the University of Alabama Medical School. The Scandinavian portion of the study is being conducted in three university towns (Bergen and Trondheim, Norway and Uppsala, Sweden), in which the medical care of pregnant women in the surrounding counties is provided for through each of the University Hospitals.

The goal of the study is to identify risk factors which will distinguish mothers having repeated small-for-gestational age (SGA) births from those who have an SGA birth unexpected from prior pregnancy history. The study populations are therefore based on the selection of high-risk samples, consisting of para 1 and 2 mothers with one or more of the following high risk characteristics:

1. previous low birth weight delivery;
2. previous perinatal death;
3. serious renal disease or hypertension during pregnancy;
4. low maternal pre-pregnancy weight (<50 kg);
5. cigarette smoking at conception.

In addition to the above-listed risk factors which are common to the protocols used at all sites, the University of Alabama protocol includes the following additional criteria as well:

6. previous spontaneous abortions (2 or more);
7. previous preterm delivery (<37 weeks);
8. low maternal height (<157 cm);
9. alcohol drinking during pregnancy;
10. late onset of first prenatal care visit (26-32 weeks).

Several statistical techniques are being used to test for significant factors affecting fetal growth, including multiple linear and logistic regression analyses.

Major Findings:

A number of manuscripts have been written, submitted and some published. They deal with the relationship between a psychological profile, maternal size and smoking and predicting intrauterine growth retardation, comparison of fetal biparietal diameter, head circumference, abdominal circumference and femur length by race and sex, the relationship between maternal blood pressure, fetal growth retardation and preterm delivery; the study of maternal psychological characteristics and intrauterine growth retardation, as well as others. A special issue of Acta Obstet Gynecol will be published in 1995 summarizing findings from the first cycle of the study. The analyses of the five year follow up data has been initiated. The most important predictor of mental retardation at five years are the social circumstances of the family. Reduction in mean IQ is also predicted by size at birth and intrauterine growth retardation. Abstracts dealing with cognitive development at 5 years were presented at the 1995 Society for Gynecologic Investigation meeting and the 1995 Society for Epidemiologic Research meetings.

Intrauterine growth curves follow a sigmoid pattern. Between 11 and 35 weeks, the growth increases in a linear fashion slow at approximately 35 weeks. Risk factors for IUGR appear to have a small effect on growth between 11 and 35 weeks, and a more marked effect on growth after 35 weeks. Growth retardation, especially a reduction in head circumference has important negative effects on cognitive development and growth.

Significance to Biomedical Research and the Program of the Institute:

Factors which affect intrauterine growth and which may provide insight into the pathophysiology of intrauterine growth retardation are of considerable significance to the mission of this Institute. Intrauterine growth retardation is a significant public health problem and associated with higher rates of perinatal infant mortality and increased morbidity.

Proposed Course:

The children are being assessed as they become five years of age. Further analysis of this data are in progress. Dr. Herman together with scientists from both contracts will examine the intrauterine dynamics of fetal growth among infants with IUGR. In addition, the role of emerging risk factors such as psychosocial stress will be explored in these data sets.

Contract Report:

The data collection prospectively during pregnancy and follow-up of infants through the first year of life was supported by the University of Alabama in Birmingham under NICHD contract N01-HD-4-2811 and by the University of Trondheim in Norway under NICHD contract N01-HD-4-2803. These two contracts entitled "Successive Small-for-Gestational Age (SGA) Births: A Longitudinal Study of Fetal Growth and Perinatal Outcome" were initiated June 1, 1984. These two contracts terminated in FY 91. The data collection for the two new contracts entitled "Follow-up Study of Mental, Physical and Behavioral Development in Five Year Old Children" with the University of Alabama in Birmingham under NICHD contract N01-HD-1-3116 was initiated march 1, 1992 and with the University of Trondheim in Norway under NICHD contact N01-HD-1-3127 was initiated April 1, 1991. In the current period (FY 96), $53,579.44 was spent on N01-HD-3127.

Publications:

Goldenberg RL, Dubard MB, Cliver SP, Nelson KG, Blankson K, Herman AA. Pregnancy outcome and intelligence at age five. Am J Obstet Gynecol (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00872-11 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Factors Associated with Premature Births: Missouri Follow-Back Survey

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A.A. Herman	Visiting Scientist	EB, DESPR, NICHD
Other:	H.W. Berendes	Director	OD, DESPR, NICHD

COOPERATING UNITS *(if any)*
NIDCD (H.J. Hoffman); State Center for Health Statistics, Missouri Dept. of Health (G. Land, W. Schramm, J. Stockbauer, V. Pierson, B. Boley) University of Missouri (L. Longo, M. Sable)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 0.2
PROFESSIONAL: 0.2
OTHER: 0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective is to obtain more accurate information relating to the very low birth weight (VLBW) infant, <1500 grams, than is now available from the United States vital records. This objective will be accomplished by the following: 1) mailing or administering a questionnaire to mothers of VLBW infants, mothers of fetal deaths, and a sample of mothers of moderately LBW infants (1500-2499 grams) and normal birth weight infants (≥2500 grams) in order to obtain and verify information from the prenatal, perinatal, and post-neonatal periods; 2) conducting telephone follow-up interviews on non-respondents and incomplete respondents, and a 10 percent sample of study mothers to obtain and/or verify information on the questionnaires; and 3) developing procedures for abstracting information from hospital and physician records, including otherwise unavailable or missing information on morbidity, lifestyle, and socioeconomic indicators of the study subjects. In addition, mortality and results of follow-up evaluations will be available through the first year of life for this birth cohort. Initial analyses of these data focused on the impact of very low birth weight and moderate low birth weight on cognitive development at one year, and the effect of maternal attitudes to weight gain on nutritional status and birth weight outcome.

Further work is being done on the impact of prenatal care (timing and content) on pregnancy outcome, the impact of poverty, socioeconomic and psychosocial factors on preterm labor and delivery and IUGR, the impact of pregnancy wantedness on pregnancy outcome, and the impact of prenatal, intrapartum and neonatal care on neonatal outcome.

Project Description:

Objectives:

This study is intended to obtain more accurate information relating to the very low birth weight infant, <1500 grams, than is not available.

Methods Employed:

This is a population-based study of all very low birth weight (VLBW) infants and fetal deaths occurring in Missouri during a 16 month time period (December 1, 1989 through March 31, 1991). Moderately low birth weight and normal birth weight controls have been matched to the VLBW infants. The Missouri Department of Health has gathered data by means of questionnaires from mothers of infants and fetal deaths, hospitals and physicians. These data were linked to birth - and infant death certificates. Surviving infants were followed up to one year of age. The revised Denver II screening examination was used to evaluate cognitive development at one year. In addition the use of well-baby health care was assessed and illnesses during the first year of life were ascertained from pediatric and maternal questionnaires.

Major Findings:

Infant mortality rates among very low birth weight infants were very high. The surviving very low birth weight infants had an increased frequency of cognitive deficits at one year. Moderately low birth weight infants had higher rates of developmental delay at one year when compared to normal birth weight infants. Women who were embarrassed about being fat gained less gestational weight that those who were proud of being pregnant. Those who did not worry about size, and those who held the baby's health of paramount importance gained more weight during pregnancy.

Significance to Biomedical Research and the Program of the Institute:

The study data will add considerably to our knowledge of risk factors and current morbidity conditions in the perinatal and post-perinatal periods. The initial results on cognitive development at one year has important implications for the impact of preterm birth and growth retardation on early schooling problems.

Proposed Course of Project:

To continue the analyses of data and design a low term follow-up study of cognitive and physical health among preterm and growth retarded infants.

Publications:

Two abstracts on risk factors for low birth weight were published at the 1994 American Public Health Association meeting.

Contract Report:

The data collection was supported by the Missouri Department of Health under NICHD contract N01-HD-6-2916. This contract entitled "Factors Associated with Premature Births: The Missouri Follow-back Survey" was initiated January 2, 1986. The final voucher for the amount of $10,118.09 was submitted by the Missouri Department of Health on 5/3/96.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02500-04 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
ETEC Epidemiology and Vaccine Research in Lower Egypt

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD
	A. Naficy	Expert	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
NAMRU-3 Unit, Cairo, Egypt (S. Savarino, R. Abu Elyzeed, B. Kay, L. Peruski); University of Alexandria (A. Mourad, M. Gaafar, M. Shasly); University of Goteborg (J. Holmgren, A-M. Svennerholm).

LAB/BRANCH
Epidemiology Branch

SECTION
Pediatric Infectious Diseases and Vaccinology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Enterotoxigenic Escherichia coli (ETEC) diarrhea is hyperendemic among young Egyptian children, with a balanced distribution of toxin phenotypes from pathogenic isolates. These features make it logical to develop a field site for the evaluation of ETEC epidemiology and ETEC vaccines in Egypt. We have begun a collaborative program to develop a field site near Alexandria for the study of pediatric ETEC diarrhea, and to evaluate the safety and immunogenicity of a promising killed oral ETEC vaccine candidate in preparation for a field trial of vaccine efficacy.

This program of projects has followed two pediatric cohorts, one in Abees (Alexandria governorate) and the other in Abu Homos (Beheira governorate) to determine the age-specific incidence rate of ETEC diarrhea from birth to 35 months, by toxin and colonization factor (CFA) phenotype; and to evaluate the protective relationship between titers of serum IgG antibodies to ETEC toxins and CFAs and the risk of diarrhea due to ETEC manifesting these virulence factors. During two years of follow-up of 242 children in Abees, the incidence of ETEC diarrhea was found to be .9 episodes per child-year, and 88% of isolates expressed LT-only or ST-only. Approximately one-fourth of cases were due to an ETEC that expressed a recognized CFA antigen. Among the 286 children followed for one-year in Abu Homos, the incidence of ETEC was 1.4 episodes per child-year, with 80% of isolates expressing LT-only or ST-only, and ca. 40% of isolates expressed a CFA.

A randomized, placebo-controlled Phase 2 study of killed oral ETEC vaccine, administered as a two-dose regimen to 76 adults in Benha, near Cairo, demonstrated the vaccine to be well-tolerated and to induce signficant musosal immune responses to vaccine antigens. A similarly designed trial of the vaccine, administered to 110 children aged 6-12 years in the same site was recently conducted. No serious reactions were noted, but definitive analyses await unblinding of the codes.

Project Description:

Personnel: Dr. John Clemens of the Epidemiology Branch, DESPR, is the principal investigator of this project, with responsibility for the epidemiological design, execution, and analysis of the study. Implementation of the study is additionally being undertaken by scientists at the NAMRU-3 Unit in Cairo (Drs. S. Savarino, R. Abu Elyzeed, B. Kay, L. Peruski) and the Departments of Microbiology (Prof. A. Mourad) and Preventive Medicine (Prof. M. Shasly) of the University of Alexandria. Professors J. Holmgren and A-M. Svennerholm of the Department of Medical Microbiology of the University of Goteborg are consulting on microbiological aspects of the study. Mr. M.R. Rao of NICHD is responsible for the overall design of computerized data management, and Dr. A. Naficy is assisting Dr. Clemens on design and analysis issues.

Objectives:

1. To determine in two different field sites the age-specific incidence rate of ETEC diarrhea from birth to 35 months, by toxin and colonization factor (CFA) phenotype.

2. To ascertain the strength of attribution of diarrheal symptoms to fecal isolation of ETEC in each site.

3. To evaluate the protective relationship between titers of serum IgG antibodies to ETEC toxins and CFAs and the risk of diarrhea due to ETEC manifesting these virulence factors in each site.

4. To establish, as a basis for future field trials of ETEC vaccines, the field, laboratory, and data management capabilities for detecting and etiologically characterizing ETEC diarrhea by toxin and CFA phenotype and for evaluating serological responses to ETEC virulence factors.

5. To evaluate the safety and immunogenicity of a killed oral ETEC vaccine, consisting of cholera toxin B subunit and strains of formalin-killed ETEC that manifest antigens of colonization factors (CFAs) I, II, and IV.

6. To assess the clinical efficacy of this killed ETEC vaccine when administered to Egyptian infants.

Methods Employed:

Two cohort studies, one conducted in Abees, Alexandria governorate, and the other in Abu Homos, Beheira governorate, have employed a common protocol. Each study has assembled a cohort of newborns and children under 24 months of age, and, with twice-weekly visits to homes, has ascertained diarrheal events, which are characterized microbiologically for ETEC (including specific ETEC virulence factors) and other conventional enteropathogens using conventional techniques. Blood is collected by fingerstick every three months, and sera will be assayed for anti-ETEC IgG antibodies, for correlation with the ensuing incidence of diarrhea caused by ETEC exhibiting homologous virulence factors.

Studies of the killed oral ETEC vaccine are being conducted in sequence, first in adults, then in older children (6-12 years), next in younger children (2-5 years), and finally in infants. Each study evaluates the safety and immunogenicity of a two-dose (separated by 14 days) regimen of the vaccine coadministered with buffer, and each study is conducted in a randomized, placebo-controlled fashion.

Major Findings:

During two years of follow-up of 242 children in Abees, the incidence of ETEC diarrhea was found to be .9 episodes per child-year, and 88% of isolates expressed LT (heat-labile toxin)-only or ST (heat stable toxin)-only. Approximately one-fourth of cases were due to an ETEC that expressed a recognized CFA antigen. Among the 286 children followed for one-year in Abu Homos, the incidence of ETEC was 1.4 episodes per child-year, with 80% of isolates expressing LT-only or ST-only, and ca. 40% of isolates expressed a CFA. Analyses of sera obtained are now in progress.

A randomized, placebo-controlled Phase 2 study of killed oral ETEC vaccine, administered as a two-dose regimen to 76 adults in Benha, near Cairo, demonstrated the vaccine to be well-tolerated and to induce signficant musosal immune responses to vaccine antigens. A similarly designed trial of the vaccine, administered to 110 children aged 6-12 years in the same site was recently conducted. No serious reactions were noted, but definitive analyses await unblinding of the codes.

Significance to Biomedical Research and the Program of the Institute:

It has been estimated that among children under 5 years in the developing world, nearly 70 million episodes of diarrheal disease due to ETEC occur each year, accounting for approximately 800,000 deaths. Although crude, these estimates highlight the high disease burden of ETEC infections in the developing world, and indicate that a vaccine which is effective against ETEC would be of great public health benefit to children in these settings. Accordingly, the development and testing of effective vaccines against ETEC diarrhea has been identified as a specific priority of the Programme for Vaccine Development of the World Health Organization. Critical to the testing of future ETEC vaccines is the development of field sites in which ETEC is endemic, and in which the epidemiology of ETEC is characterized in suitable detail for estimation of sample size requirements and other aspects of study design. In this regard, it is important that the occurrence of ETEC by toxin and CFA phenotype be known, since vaccine protection is expected to be homologous with respect to these virulence factors, and since the distribution of these factors is known to vary substantially in different areas.

The present research is being conducted in Abees and Abu Homos, Egypt, near Alexandria, as well as in Benha, near Cairo. This research is being conducted as a collaboration between the National Institute of Child Health and Human Development (NICHD) of the U.S. National Institutes of Health; the U.S. Navy, through the research unit in Cairo, Egypt (U.S. Navy Medical Research Unit No. 3: NAMRU-3); and the Faculty of Medicine, Alexandria University, Alexandria, Egypt (Departments of Microbiology and Community Medicine). It is anticipated that the Abu Homos field site will ultimately serve as a site for evaluation of killed oral vaccine against ETEC. In addition to providing important epidemiological information about childhood ETEC diarrhea, epidemiological work in Abu Homos this study will augment the field and laboratory infrastructure of this field site, so that it will be suitable for the testing of vaccines against ETEC as well as future vaccines against other pediatric enteric pathogens.

Proposed Course: This is envisioned as a four-year project.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02502-04 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
NICHD-Health Research Board of Ireland Neural Tube Defects Study

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.L. Mills	Chief, Pediatric Epidemiology Section	EB, DESPR, NICHD
Other:	M.R. Conley	Computer Specialist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Health Research Board of Ireland and Trinity College, Ireland

LAB/BRANCH
Epidemiology Branch

SECTION
Pediatric Epidemiology Section

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Epidemiology Branch (DESPR) is conducting a number of studies in collaboration with the Health Research Board and Trinity College, Ireland. These investigations are designed to determine the biochemical mechanisms by which folate reduces the risk for neural tube defects. Data and blood samples have been collected on a large proportion of Irish women delivering babies in Dublin. Samples from women whose pregnancy ended in the delivery of a child with a neural tube defect and control women whose pregnancy ended in the delivery of a normal child are being studied. Samples are also being collected from families with an affected child. Various aspects of folate metabolism and other nutritional measures are being examined. We have demonstrated that at lower levels of B12, women carrying a fetus with an NTD have significantly higher levels of homocysteine than women carrying a normal fetus. We have also demonstrated that an abnormal gene responsible for elimination of homocysteine is involved. Specifically, a gene defect produces the so-called thermolabile variant of the 5,10 methylene tetrahydrofolate reductase enzyme. This abnormal enzyme is found significantly more frequently in individuals with NTDs than in normal individuals.

Project Description:

Dr. James Mills of the Epidemiology Branch, DESPR directs the NICHD-Health Research Board of Ireland Neural Tube Defects study. Subjects for this study have been identified in the Dublin area by our collaborators in the Health Research Board under the direction of Dr. Peadar Kirke. A biochemical investigation is being done in the laboratory of Professor John Scott at Trinity College, Dublin. Ms. Mary Conley of the DESPR is responsible for data center and Dr. Jack Lee of the DESPR is the statistician.

Objectives:

To identify the mechanism by which folate prevents neural tube defects, and to characterize the population at risk.

Methods Employed:

Ongoing biochemical assays are looking for differences in mothers who have had NTD children both during affected pregnancies and at other times. Genetic studies to identify markers for abnormal enzyme function are also ongoing.

Major Findings:

Having demonstrated abnormal homocysteine metabolism in mothers carrying neural tube defect offspring, we have investigated some possible enzyme defects that could cause abnormal homocysteine metabolism. A defect in the gene for the methylene tetrahydrofolate reductase enzyme has been shown to produce a less efficient enzyme. This abnormal enzyme is present significantly more often in NTD cases than normal controls.

Significance to Biomedical Research and the Program of the Institute:

We have taken an important step in identifying an initial genetic component which acts with environmental components (folic acid and vitamin B12 levels) to produce neural tube defects. If it becomes possible to identify women at risk using markers for abnormal enzyme function, it will be possible to target vitamin prophylaxis. This would be of significant clinical and economic benefit.

Proposed Course:

Current plans include further genetic studies in search of other abnormalities explaining more NTD cases. We will continue to study mothers of NTD children during an affected pregnancy, a non-affected pregnancy, and in a non-pregnant state. We are developing cell culture techniques to study enzyme function directly. The folic acid dose and duration of therapy necessary to raise a sub-optimal red cell folate level to an optimal plateau is now being studied in the female employees of a Dublin hospital.

Publications:

Mills JL, Scott JM, Kirke PN, McPartlin JM, Conley MR, Weir DG, Molloy AM, Lee YJ. Homocysteine and neural tube defects, J Nutr 1996;126:756S-60S.

Whitehead AS, Gallagher P, Mills JL, Kirke PN, Burke H, Molloy AM, Weir DG, Shields DC, Scott JM. A genetic defect in 5,10 methylenetetrahydro-folate reductase in neural tube defects, Q J Med 1995;88:763-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02506-04 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Lack of Age-Appropriate Immunization among Infants Born in District of Columbia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. Brenner	Staff Fellow	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief	EB, DESPR, NICHD
	B. Simons-Morton	Health Research Specialist	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Children's National Medical Center; D.C. Commission of Public Health; D.C. General Hospital; Georgetown University; Howard University; University of the District of Columbia

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As part of the "NIH/DC Initiative to Reduce Infant Mortality" a program of research aimed at increasing the proportion of infants and children in the District of Columbia who are age-appropriately immunized has been developed. This program of research includes both epidemiologic and intervention (efficacy) studies. The phase I studies include needs assessment and feasibility studies in addition to a study examining determinants (as measured at birth) of immunization status at 3 and 7 months of age. The findings from this phase will be used in the planning and execution of an intervention study in phase II.

Investigators include epidemiologists, behavioral scientists, health care providers and administrators. The program of research was developed with input from the variety of perspectives represented by the investigators and the proposed studies have the potential to benefit from a unique blend of epidemiology, behavioral science, and public health and health care practice. It is our intent to develop interventions and identify determinants that are relevant to public health practice in inner cities across the US.

This report focuses on the phase I epidemiologic studies.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

1) Identify prospectively (at the time of birth) determinants of age-appropriate immunization among infants and children born to residents of the District of Columbia.

2) Assess the association between lack of age-appropriate immunization and other poor health outcomes.

Methods Employed:

A birth cohort will be assembled from nursery log books. Baseline information will be gathered via face to face interviews with the mother during her hospital stay or, if this is not feasible, within two weeks of the delivery.

Demographic information as well as "consumer characteristics" (e.g. knowledge, attitudes, and beliefs concerning immunizations, well child care and childhood illness) will be collected at this time. Infants/Mothers will be contacted at 3 months and at 7 months to assess immunization status and other health outcomes. Medical records will be reviewed for all health care visits. Information regarding health care provider characteristics and health care system characteristics will be gathered for all identified sites of patient care. (Methods for determining the current residence of infants and for retrospectively ascertaining immunization status and other health outcomes will also be addressed in the phase I studies.)

To determine predictors of delayed initiation of immunization, infants who are appropriately immunized at three months of age will be contrasted to those who are not. Similarly, to determine predictors of attenuation of age-appropriate immunization status, infants who are initially immunized at 2 months of age but then fail to complete the primary series in a timely fashion will be contrasted with infants who are appropriately immunized at 7 months.

Major Findings:

Data not yet available for analysis.

Significance to Biomedical Research and the Program of the Institute:

Several factors make this an extremely important project: 1) Assurance of age-appropriate immunization is a national public health objective. The National Health Promotion and Disease Prevention Objectives (Healthy People 2000) include a national goal of at least 90% completion of the recommended immunizations by 24 months of age. 2) Surveys conducted in the District of Columbia in 1990 and 1991 revealed that less than 40% of children had received all recommended immunizations by 2 years of age. 3) Immunization is only one of many important components of preventive pediatric health care for infants and children. Interventions to improve immunization status may have the added benefit of increasing utilization of preventive health care, linking infants and children to systems that may provide continuity of care. 4) Studies in other cities have identified delayed receipt of the initial two month vaccine as the strongest risk factor for lack of age appropriate immunization at two years. This study will be one of the first to prospectively examine factors related to delayed initiation of immunization. In addition, it is desirable to identify determinants of immunization status prospectively (at the time of delivery) such that high risk families can be identified for targeted interventions.

Proposed Course of Project:

The hospital-based cohort study will be conducted over a twenty-four month period. Study enrollment began in August of 1995 and will continue through August 1996. Based on these recruitment projections, the 3 and 7 month interviews will be completed by the Spring of 1997 and medical record reviews will be completed by August of 1997.

Publications: None yet.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02507-04 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Prostaglandin Excretion in Preeclampsia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.L. Mills	Chief, PES	EB, DESPR, NICHD
Other:	R.J. Levine	Expert	EB, DESPR, NICHD
	J.D. Clemens	Chief, EB	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A disturbance of prostaglandin metabolism is currently believed to be one of the major mechanisms in the pathogenesis of preeclampsia. Most previous studies of this theory, however, have included only women who had already developed clinically overt disease. In order to investigate whether prostaglandin abnormalities precede the onset of preeclampsia, we have designed a nested case-control study using stored urine specimens prospectively collected as early as thirteen weeks gestation from women participating in the NICHD Trial of Calcium to Prevent Preeclampsia (CPEP). In this study, we will compare the urinary excretion of prostaglandin and thromboxane in preeclampsic women before the diagnosis of preeclampsia to that in normal pregnant women at comparable gestational ages. Because half of the women in the CPEP trial will be taking two grams of oral calcium daily, we will also be able to examine the effect of oral calcium supplementation on the excretion of these prostaglandin metabolites.

Project Description:

Dr. James Mills, Dr. Richard Levine, and Dr. John Clemens are collaborating on this project. The laboratory analyses will be performed by Dr. Jackson Roberts at Vanderbilt University.

Objectives:

To determine whether an imbalance of prostaglandin metabolism precedes the clinical onset of preeclampsia.

Methods Employed:

Vanderbilt University is assaying specimens (blinded to status) from women who developed preeclampsia and normal control women. Thus far, nearly 200 urine specimens have been assayed for the prostacyclin metabolite 2,3-dinor-6-keto-$PGF_{1\ alpha}$ and the thromboxane metabolite 11-dehydro-TxB_2.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute:

Preeclampsia occurs in 5-10% of all pregnancies, and causes substantial maternal and neonatal morbidity. Determining the pathogenic mechanisms underlying this disease will assist in development of rational prediction tests and therapies.

Proposed Course:

The last cases of preeclampsia in the CPEP study have been identified. Once they and appropriate control subjects have urine specimens transported to Vanderbilt, the final assays can be performed. Then the codes can be broken and the analyses started.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02510-03 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Diet, Maternal Nutritional Status, Blood Pressure and Fetal Growth

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Tafari	Visiting Scientist	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief	EB, DESPR, NICHD
	H.W. Berendes	Director	DESPR, NICHD
	Y. Johnson	IRTA Fellow	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Ethiopian Nutrition Institute (Z.W. Gabriel, E. Woohib)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The project has been terminated.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02511-02 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunogenicity of Routine Childhood Vaccines in HIV Positive Children

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.S. Read	Senior Staff Fellow	EB, DESPR, NICHD
Other:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
FDA (B. Anthony); NERI (D. Brambilla); NIAID (M.G. Fowler); Columbia University (J. Pitt); University of Illinois (K. Rich)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*
This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02512-03 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Birth Certificate Linkage to Growth and Health Measures using NHANESIII

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*

National Center for Health Statistics (R. Kuczmarski, G. Gay, K. Maurer); Centers for Disease Control (R. Etzel); NIAID (P. Gergen)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.25	.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The third National Health and Nutrition Examination Survey (NHANESIII) has collected descriptive data, physical measures, and biomedical samples (blood and urine) on a representative U.S. sample of children at ages two months through six years with oversampling for blacks and Mexican Americans. Serum cotinine levels were measured and information on prenatal maternal smoking obtained. Linkage of the extensive data from NHANESIII to the child's birth certificate to use the prenatal and birth information has begun. All of the states were sent requests for the relevant birth certificates with the signed permission of parents of the examined child. All certificates have been received. Computerized matching of NHANESIII and birth certificate data is underway.

Results of the linkage will support research efforts to do the following: 1) To determine if low birth weight or premature children have subsequent poor health as measured by biomedical markers in a representative national sample of children through age six; 2) to assess the effects of ethnic/racial and socioeconomic factors on these outcomes; 3) to develop normative growth and other biomedical scales for a U.S. representative population, and black and Mexican-American children, according to a risk profile at birth; and 4) to develop comparisons between maternal and proxy reports of children's risk factors at birth and actual measurements for use in other studies.

Analysis has begun on ear infections and respiratory outcomes in children associated with prenatal maternal smoking and current household exposures measured by serum cotinine levels.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

1) To determine if low birthweight or premature children have subsequent poor health as measured by biomedical markers in a representative national sample of children through age six.

2) to assess the effects of ethnic/racial and socioeconomic factors on these outcomes.

3) to develop normative growth and other biomedical scales for a U.S. representative population, and black and Mexican-American children, according to a risk profile at birth.

4) to develop comparisons between maternal and proxy reports of children's risk factors at birth and actual measurements for use in other studies. Major outcomes of interest will include 1) weight and height growth scales by risk profiles at birth; 2) effects of passive smoke exposure in the household controlling for prenatal factors; and 3) other health measures collected both by questionnaire and biomedical measurement.

Methods Employed:

The third National Health and Nutrition Examination Survey (NHANES III) has collected descriptive data, physical measures, and biomedical samples on approximately 9,000 children at ages two months through six years with oversampling for blacks and Mexican Americans. The extensive data from NHANES III will be linked to the child's birth certificate to use the prenatal and birth information. NCHS has obtained the certificates from the States and is linking the data. Data analysis will require application of appropriate software for analysis of the complex multistage sample design of NHANES III for both descriptive and multivariate findings.

Major Findings:

None available yet.

Significance to Biomedical Research and the Program of the Institute:

Our ability to determine the health outcome of children classified as high risk at birth compared to other children has been extremely limited. Children who have been followed are seldom representative of even small areas. Normative comparisons for hypothesis testing of poor health outcomes have been based on scales developed from small, potentially biased populations. Use of this nationally representative sample including growth and other biomedical measures is a unique opportunity to provide norms for both the clinical and research community.

Proposed Course:

We will perform both descriptive and multivariate predictive analysis for publication in peer-reviewed journals. Analysis with serum cotinine levels has begun. Birth certificate linkage to NHANES files is underway to begin analysis of birth weight-specific profiles.

Publications: None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02513-03 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Survival Status and Risk Factors Analysis of Premature High-Risk Deliveries

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD
Other:	A.C. Trumble	Chief, Computer Sciences Branch	CSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

March of Dimes (J. Petrini, K. Damus)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Trends toward increasing survival of infants born at less than 750 grams (about 1-1/2 pounds) have suggested higher prevalence of complications related to birth and continuing health deficits. Dr. Overpeck documented the status for the births, subsequent deaths, and survival in the U.S. birth cohort of 1983. She has provided background and technological support for staff of the March of Dimes (Joann Petrini) to complete the collaborative analysis to assess changes in survival and mortality of these extremely low birth weight infants. The analysis will compare the baseline years before introduction of surfactant therapy (1983), years incorporating the experimental phase of surfactant therapy (1987-88), and the latest years of available data which include births associated with both surfactant and antenatal corticosteroid therapy (1990-92). Deaths to infants born in the baseline years at weights less than 750 grams (about 24 weeks gestation) contributed about 25 percent of total infant mortality in 1983. Over 40 percent of deaths prior to six days of life were from these smallest least mature infants.

Project Description

Objectives:

To assess changes in survival and mortality of live-born infants weighing less than 750 grams in the U.S.

Methods Employed:

Summary data prepared by NCHS for release with the public use tapes of the 1983 Birth Cohort Linked Birth/Infant Data Set were analyzed for U.S. resident deaths of infants born in 1983. Data from 1983, 1988, and 1991 are being compared for the total U.S. and local areas. Multivariate risk factor analysis will be completed.

Major Findings:

Deaths in infants born at less than 500 grams (about 20 weeks gestation) contributed about 10 percent of total infant mortality in 1983. Deaths to infants born at less than 750 grams contributed about 25 percent. About 33 percent of all Black deaths occur to infants born at less than 750 grams; 22 percent of White deaths occur in this birth weight range. Over 40 percent of deaths prior to six days of life are from these smallest, least mature infants. Decreases in death rates are occurring for births >750 grams but not in the highest risk weights ≤750 grams.

Significance to Biomedical Research and the Program of the Institute:

The analysis of birth weight-specific mortality rates focuses attention on the timing of interventions needed to reduce infant mortality and to prevent such high risk deliveries. Birth weight-specific comparisons at the national and local levels will be made to assess differences in risk factors and outcomes. The time periods analyzed compare years encompassing the phases of testing and use of both surfactant and antenatal corticosteroid therapy. Changes in death rates by birth weight indicate that effects of surfactant therapy are specific to infant weights and gestations at risk for respiratory distress syndrome but not for those with very incomplete lung development.

Proposed Course:

Changes in survival rates since 1983 are being analyzed with multivariate risk factor assessment. Publication in peer-reviewed journals and dissemination of local data through the March of Dimes is expected.

Publications: None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 02514-03 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Childhood Drowning Deaths - A National Analysis of Death Certificate Information

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.A. Brenner	Staff Fellow	EB, DESPR, NICHD
Other:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Johns Hopkins University (G. Smith); Consumer Product Safety Commission (A. McDonald)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Working in collaboration with the Consumer Product Safety Commission (CPSC) information is being abstracted from death certificates for all non-boat related drowning deaths in children and adolescents less than 20 years of age. The free text portion of the death certificate often includes important information that is not included in national mortality files. Of particular importance is the specific site of the occurrence (e.g. swimming pool, lake, irrigation ditch, etc.) as interventions to prevent drowning deaths are site specific. Results from this study will provide the first in depth look at the circumstances surrounding drowning deaths at a national level.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

To describe the epidemiology of drowning in the U.S. through the abstraction and analysis of death certificate information.

Methods Employed:

Through an agreement with the Consumer Product Safety Commission, death certificates will be purchased from the fifty states and two major health jurisdictions (New York City and Washington, D.C.) for deaths in which the underlying cause of death is a nonboat-related drowning (E-code 910.0-910.9, 954, 964 and 984) and the age of the victim at the time of death is less than 20 years. Information from the purchased death certificates will be abstracted and coded to provide a uniform dataset for analyses of drowning deaths.

Data will be used in descriptive analyses of the circumstances of drowning, with particular focus on stratified analyses of the specific site of drowning by age at death and state of occurrence. Other variables of interest include sex, race, products associated with the death, and other details surrounding the event (e.g. the involvement of alcohol) abstracted from the narrative portion of the death certificate .

Major Findings:

Results not yet available.

Significance to Biomedical Research and the Program of the Institute:

In the United States, drowning is the second most common cause of unintentional injury death among children and adolescents. In the one to two year old age group it is the single leading cause of injury death. Other groups at increased risk of drowning include adolescent males (particularly black adolescent males) and, in recent years, infants less than one year of age.

Interventions to prevent drowning deaths are dependent not only on the age of the victim but also on the specific location and circumstances surrounding the event. Unfortunately, national mortality data often do not provide sufficient information regarding the circumstances of these drowning deaths to allow injury prevention experts to design appropriately targeted interventions. In particular the specific site of the drowning (i.e. swimming pool, lake, bucket, irrigation ditch etc.) can not be identified from computerized mortality data because the E-Code (the ICD code for external injuries) is not specific for site of drowning. The hard copy of death certificates are a valuable source of information on the circumstances surrounding drowning deaths, including the specific site of the event.

This study will be the first to describe, at a national level, the specific circumstances surrounding drowning deaths thus enabling the development of appropriately targeted interventions.

Proposed Course of Project:

As outlined in the interagency agreement with CPSC, death certificates for all drowning deaths received since January 1, 1994 have been retained by CPSC. CPSC has coded information from those drowning deaths that were not product related. (Coding of information from product related deaths is in the usual scope of work at CPSC.) Contracts with the 50 states and two major health jurisdictions (New York City and Washington, DC) for the purchase of death certificates have been modified to include all non-boat related drowning deaths as outlined in the interagency agreement. These modifications took effect in FY'95. As of December 1995, 1008 death certificates were abstracted for deaths occurring in 1993, 817 for 1994 and 196 for 1995. Under the terms of the agreement, CPSC will continue coding death certificate information for deaths occurring through December 31, 1995. It is anticipated that the most complete dataset will be compiled for the 1994 and 1995 calendar years.

Publications: Listed last year.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02515-03 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prevention of Childhood Injuries - Phase I Injury Surveillance

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.A. Brenner	Staff Fellow	EB, DESPR, NICHD
Other:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Children's National Medical Center (P. Scheidt); D.C. Commission of Public Health; D.C. General Hospital; Georgetown University; Howard University; University of the District of Columbia

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As one component of the "NIH/DC Initiative to Reduce Infant Mortality" a program of research addressing the threat of injuries and neglect to infants and toddlers in the District of Columbia has been developed. The study is divided into two phases. During phase one a comprehensive, city-wide injury surveillance system will be established. This surveillance will include all injuries in the target age group (birth through two years) that result in an emergency room visit, hospitalization or death. Information will also be obtained from the Department of Family Services on substantiated cases of abuse and neglect.

The information obtained in phase I will guide the design of specific targeted interventions, for the prevention of injuries, that will be implemented and evaluated in phase II. A randomized intervention trial of local community entities (neighborhoods or wards) is proposed in which epidemiologically based environmental interventions are applied to one group, behaviorally based interventions to prevent abuse and neglect to a second group and epidemiologic surveillance alone to a third group. As an essential component of injury prevention, the injury surveillance system will continue for all three groups, throughout phase II.

The remainder of this report focuses on the phase I surveillance activities as detailed plans for the intervention phase are dependent on the results of the surveillance and thus have not yet been developed.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

1) To collect citywide epidemiological data to define causes and patterns of injuries, both intentional and unintentional, that will enable investigators to develop effective intervention approaches in phase II of this study.

2) To determine the direct costs of intentional and unintentional injuries in the target population.

Methods Employed:

Information will be abstracted from medical records and billing records of children ages 0-2 years who were seen in an emergency room or admitted to a hospital for an injury. All non-military hospitals in which children from the District of Columbia routinely seek care will be included in the surveillance. In addition, at three study sites a questionnaire will be administered to the parent or caretaker at the time of the ER visit or hospitalization. This questionnaire includes information on circumstances surrounding the injury event and other potential risk factors. Intake records from the Department of Family Services will be abstracted to provide information on cases of substantiated child abuse and neglect.

The leading causes of fatal and nonfatal injuries will be identified. Emergency room injury incidence rates, hospital admission rates and fatal injury incidence rates will be calculated. Injury rates will be compared between census tracts to identify communities in which children are at increased risk. Where community data exists on sociodemographic characteristics (e.g. maternal age) analyses will compare the characteristics of the injured population to those of the community to identify characteristics which place various subgroups at increased risk of injury.

Major Findings:

Data not yet available for analysis.

Significance to Biomedical Research and the Program of the Institute:

Injuries are the third leading cause of postneonatal death and the leading cause of death after the first year of life. Studies have shown that many of these injuries are preventable. Another serious problem is that of child neglect. In 1992, the D.C. Commission of Social Services investigated 4,886 families (with a total of 10,922 children and infants) for neglect. Approximately 25% of reported cases involve children less than three year of age.

The "NIH/DC Initiative to Reduce Infant Mortality" is aimed at addressing multiple causes and factors related to the high infant mortality rate in the District of Columbia. Among the types of research called for by the initiative is the development of interventions to prevent or reduce injuries. If interventions in phase II are effective, this program of research will not only reduce injuries among infants and young children of the District of Columbia but will also serve as a model program for other inner city communities.

Proposed Course:

Data collection for the Phase I study began in September of 1995 and will continue through September of 1996. A detailed protocol of the Phase II intervention study will be developed based on the results of the injury surveillance data.

Publications: None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02516-03 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Role of Bathtub Rings and Seats in Infant Drowning Deaths

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.A. Brenner	Staff Fellow	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Consumer Product Safety Commission (R. Rauchschwalbe); John Hopkins University (G. Smith)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Drowning is a leading cause of injury death among children in the United States. Recent studies have shown that, unlike other age groups, rates of death from drowning are actually increasing among infants. Approximately 40% of infant drownings occur in the bathtub. In this study we examine the involvement of bath rings and bath seats (products intended to support an infant in the sitting position during bathing) in drownings among infants and toddlers in the United States.

Using data from the Consumer Product Safety Commission, 27 drowning deaths, in which a bath seat or bath ring was in use at the time of submersion, were identified. Infants ranged in age from 5-15 months with a median age of 8 months. In a series of focus group discussions, parents reported that they were more likely to leave a child unattended momentarily in the bathtub if the infant was contained in a bath seat or ring. There was a reported lapse in adult supervision in 27 of the 30 (90%) incidents in which the circumstances surrounding the event are known. This study is the first in the medical literature alerting health professionals to the potential hazards of these products and reinforces the need to counsel parents on the dangers of leaving small children and infants unattended in the bathtub.

Project Description:

Objectives:

To describe drowning and near drowning incidents involving the use of bathtub rings or seats.

Methods Employed:

Data were obtained from the Consumer Product Safety Commission (CPSC) on deaths and near drownings involving bathtub seats/rings. Incidents were reported to CPSC through a number of sources including: both a voluntary and paid Medical Examiner's and Coroner's Alert Program; reports from a randomly selected number of hospitals that report all product-related emergency room injuries (NEISS); death certificates for product related incidents which are sent in from each of the fifty states and two major health jurisdictions (New York City and Washington, DC); a newspaper clipping service and a toll-free 800 line. Bathtub seat/ring incidents identified by CPSC Epidemiology staff are referred to the Division of Corrective Actions for in-depth investigation (IDI) assignment.

Reported incidents that occurred on or before December 31, 1995 and for which the IDI was completed by July 1, 1996 are included in this study. To be eligible for inclusion in this case series a bathtub seat had to be in use at the time of death or injury and the primary cause of death or injury had to be due to drowning or near drowning. Major findings are summarized below.

Major Findings:

1) Twenty-seven deaths and six near drownings were identified. The first incident occurred in August of 1983 and the most recent occurred in August of 1995.

2) The age at death ranged from 5 to 15 months, with a median age of 8 months.

3) There was generally a history of a lapse in adult supervision (either leaving the infant alone in the tub or in the care of an older child.) Among the fatal incidents, the lapse in adult supervision ranged from 0-35 minutes with a median of 5 minutes and a mean of 5.9 minutes.

4) Results from three separate focus panels showed that there is a false sense of security with bath rings and seats in that mothers/caregivers are more likely to feel comfortable leaving the infant alone in the tub for a few minutes when these products are in use.

Significance to Biomedical Research and the Program of the Institute:

Drowning is an important preventable cause of death in childhood. Among infants and toddlers a large number of these drowning deaths occur in the bathtub. This study is the first in the medical literature to identify the involvement of bath rings and bath seats in drownings among infants and toddlers. It is hoped that, once alerted to the problem, health care professionals will warn parents and caregivers about the potential hazards of these products. Additionally it is thought that there is currently substantial underreporting of incidents involving these products. It is anticipated that after results from this study are published reporting of events (particularly non fatal events) involving these products will increase giving a more accurate estimate of the extent of the problem.

Proposed Course of Project:

Preliminary findings were reported at the combined Society for Pediatric Research/American Pediatric Society/Ambulatory Pediatric Association meetings in May of 1995. A manuscript has been prepared and submitted for peer review.

Publications: None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 02517-02 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

First Week Changes in Birthweight and Other Neonatal Anthropometric Parameters

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Tafari	Visiting Scientist	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief	EB, DESPR, NICHD
	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD
	J.F. Troendle	Staff Fellow	BMSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Department of Pediatrics, Addis Ababa University, Addis Ababa, Ethiopia

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.30	0.30	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02518-02 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effectiveness of Rapid Plasma Reagin Screening in Gestational Syphilis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Tafari	Visiting Scientist	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief	EB, DESPR, NICHD
	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
National Health Research Institute, Addis Ababa, Ethiopia (D. Zewdie)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.60	0.60	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*
This project has been terminated.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02519-02 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Determination of Protective Levels of Maternal Antibody to Group B Streptococcus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	F.-Y. Lin	Research Medical Officer	EB, DESPR, NICHD
Others:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
	R.A. Brenner	Senior Staff Fellow	EB, DESPR, NICHD
	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
University of Alabama (B. Gray); Baylor College of Medicine (L. Weisman); Columbia University (J. Regan); University of Florida (P. Clark); UMDNJ (G.G. Rhoads); Magee Womens Hospital (B. Brozanski); Oakland Children's Hospital (P. Azimi)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Group B Streptococcus (GBS) is the leading cause of neonatal sepsis and meningitis. Neonatal GBS diseases occur in two forms: early-onset and late-onset. Early-onset neonatal GBS disease occurs within 7 days of life, and the late-onset disease occurs after 7 days of life. Prevention of neonatal GBS through vaccination can only be achieved through maternal transfer of antibodies to newborn infants. GBS capsular polysaccharide (Ia, Ib, II, III, IV, V) is a virulence factor and protective antigen. Vaccines based on capsular polysaccharide and protein conjugation are being developed at NICHD as well as other laboratories. Determination of a protective serum antibody threshold would provide an important target for which candidate GBS vaccines administered during pregnancy should aim.

The major objective of the study is to evaluate, for each GBS serotype, the pattern of the protective association between naturally acquired, serotype-homologous maternal serum IgG antibody titers and the risk of development of early-onset GBS disease.

The surveillance began as of July 1995. The target is to accrue 140 early-onset GBS cases and 1120 controls (colonized infants) during the 30 month study.

Project Description

Objectives:

1) The objective of this study is to evaluate the protective relationship between titers of maternal antibodies at delivery and the risk of Early-onset Group B Streptococcal (GBS) disease caused by GBS serotypes Ia, Ib, II, III, and V.

2) A secondary objective is to explore the relationship between GBS antibody titers in umbilical cord sera and the risk of early-onset disease, to assess the modifying role of placental transfer of antibodies.

Methods Employed:

Two groups of infants are being recruited in this study: infants who have developed invasive GBS disease ("cases") and infants who have not developed disease despite being surface colonized ("controls"). This study is being conducted at 13 hospitals affiliated with 7 U.S. academic institutions.

Cases are identified through active surveillance procedures established at each of the study sites. Contemporaneous controls are assembled via surface cultures obtained from the throat, anus, umbilicus and ear canal on a systematic sample of infants born at these same sites.

Maternal and cord sera are collected from these infants and their mothers. GBS isolates from case infants and from control mothers are being serotyped. Titers of IgG anti-CP antibodies to the infecting GBS serotype will be measured in maternal and cord sera, using a standardized assay. To characterize potentially confounding variables, patient charts are being reviewed. Cases and controls will be contrasted for the presence of antibody at various titers, both in simple analyses and in multivariable analyses that control for potentially confounding variables.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute:

Establishment of a protective threshold of maternal antibody to serotype-specific polysaccharide of GBS will provide an important target for which candidate GBS vaccines (a vaccine that is being developed in the Institute) administered during pregnancy should aim.

Proposed Course:

The surveillance began in July 1995 and will continue through January of 1998.

Publications: None yet.

Contract Report:

The study is conducted in collaboration with the University of Alabama in Birmingham, Baylor College of Medicine, Columbia University, University of

Florida, Magee Womens Hospital of University of Pittsburgh, New Jersey School of Medicine and Dentistry and Children's Hospital Medical Center of Northern California under the following contracts: N01-HD-4-3214, N01-HD-4-3215, N01-HD-4-3216, N01-HD-4-3217, N01-HD-4-3218, N01-HD-4-3219, and N01-HD-4-3220. The data management for the project is supported by Westat that serves as a data coordinating center under contract number N01-HD-5-3233. The 7 clinical centers are contracted to enroll GBS cases and colonized control infants. The case accrual began in July 1995. A total of 50 cases of early-onset GBS neonatal disease have been enrolled during a period of 12 months. This is slightly behind the expected number of accrual which was due to an unexpected low rate of neonatal GBS disease at one site. The contract with Magee Womens Hospital of University of Pittsburgh was terminated in June 1996 for the convenience of the Government. During FY 96, $1,185,203 has been allotted to the clinical centers, and $300,188 to the data coordinating center.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02520-02 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Maternal Caffeine Use in Pregnancy Outcome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M.A. Klebanoff	Research Medical Officer	EB, DESPR, NICHD
Others:	Richard Levine	Research Medical Officer	EB, DESPR, NICHD
	John Clemens	Chief, Epidemiology Branch	EB, DESPR, NICHD
	Rebecca DerSimonian	Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Center for Human Toxicology, University of Utah (D. Wilkins)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of maternal caffeine consumption in the pathogenesis of adverse pregnancy outcomes in controversial. Several studies have found that women who consume caffeine are at increased risk of spontaneous abortion and fetal growth retardation compared to non users. However, other, equally well-done studies have found no harmful effects of caffeine consumption. In addition, several studies have reported that caffeine is harmful only among women who smoke.

All previous studies of this question have relied on maternally-reported caffeine use; no studies have employed a biomarker for caffeine. This project will first validate the use of serum caffeine and its metabolites as a marker for caffeine intake, and will then study these serum markers as a risk factor for adverse pregnancy outcome.

In the validation study, serum paraxanthine was determined to be an acceptable marker for caffeine intake, and the main part of this project will commence in FY 97.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

1. To determine whether serum levels of caffeine and/or its primary metabolites are correlated in a useful manner with caffeine intake.

2. To determine whether maternal caffeine use during pregnancy, as determined by serum levels of caffeine and/or its primary metabolites, is associated with spontaneous abortion.

3. To determine whether maternal caffeine use, as determined by serum levels of caffeine and/or its primary metabolites, is associated with reduced fetal growth.

4. To determine if increasing maternal caffeine consumption during pregnancy as determined by serum levels of caffeine and/or its primary metabolites, is associated with less favorable neuro-development in the offspring.

5. To determine if caffeine is more deleterious in smokers compared to non-smokers.

Methods Employed:

In the first phase, the utility of serum caffeine and in paraxanthine to serve as a biomarker for caffeine intake will be evaluated. Women who completed a 24-hour dietary recall and had blood drawn at an unspecified time during working hours will be studied. The women were selected based upon their reported smoking and caffeine use. Thirty women were chosen at random from within each of 8 strata defined by 1) smoker/non-smoker and 2) caffeine consumption of <0.8 mg/kg a day; 0.8-1.49 mg/kg a day; 1.50-2.99 mg/kg a day; and $\geq$3.0 mg/kg a day.

Should the pilot data indicate that serum markers are useful, the main study will be done. In this study the first serum obtained from women experiencing a spontaneous abortion will be compared to serum obtained from women experiencing a live birth. The controls will be matched to the cases on age, time in pregnancy when the serum was drawn, study center, and smoking status. In addition, among controls, the first serum drawn after 26 weeks gestation will be evaluated to determine the association between caffeine and fetal growth retardation. Final models will include interaction times between caffeine and smoking. The exact marker for caffeine intake will depend on the pilot study results.

Major Findings:

In the pilot study, serum paraxanthine was found to be an acceptable surrogate for caffeine intake. The correlation between serum paraxanthine and caffeine intake was 0.53 in non-smokers and 0.50 in smokers. The weighted kappa between quartiles of caffeine intake and quartiles of serum paraxanthine was approximately .5. Such values are typical in nutritional epidemiology and indicate useable correlations.

Significance to Biomedical Research and the Program of the Institute:

Caffeine is the most commonly used drug during pregnancy. Between coffee, tea, chocolate and soft drinks, over half of all pregnant women are exposed to caffeine. If caffeine truly is harmful, then pregnant women should be encouraged to cut back or quit. If it is not harmful, then women may be reassured that they may safely continue its use. If caffeine is harmful only among smokers, then those women who do not smoke may be reassured, and those who smoke will have another reason to quit smoking.

Proposed Course of Project:

The data from the validation study are being prepared for publication. The laboratory analysis for the main study is scheduled to commence in FY 97. Serum from approximately 700 women with miscarriages and 2800 matched controls will be analyzed for caffeine and paraxanthine. In addition, third-trimester serum from the control women will be utilized to evaluate the association between caffeine intake and reduced fetal growth.

Publications: None yet

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02521-02 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Replacement of Pregnancy Losses: Interpregnancy Interval in Adolescence

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD
Others:	K.F. Yu	Statistician	BMSB, DESPR, NICHD
	P.M. Moyer	Computer Specialist	CSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

NIDCD (H.J. Hoffman)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Adolescent age at first pregnancy is one of the single most important risk factors for adverse pregnancy outcomes. Adolescent women have higher rates of abortion, often this is utilized as a means of contraception. Adolescents also have a higher frequency of low birth weight and preterm births, as well as fetal and neonatal death(s). The important risk factors which have been shown to be associated with poor pregnancy outcomes in adolescents include biological and behavioral factors: inadequate prenatal care, maternal smoking, maternal drug and alcohol use, poor pregravid nutritional status, acute/chronic medical conditions, inadequate education, as well as shortened spacing (<two years) between successive pregnancies.

Shortened interpregnancy intervals increase the risk of adverse pregnancy outcomes. The association between birth spacing and pregnancy outcome may be related to biological factors such as, decreased uterine tonicity, depleted maternal nutritional reserves, and inadequate time to achieve physiological equilibrium prior to the next conception. One phenomenon which occurs in older women of reproductive age, and is associated with shortened interpregnancy intervals is reproductive compensation. This occurs as a result of perinatal loss in the previous pregnancy and the desire for women to replace that loss sooner.

The purpose of this study is to evaluate the relationship between prior perinatal loss (abortion, fetal and neonatal death) and the time between successive pregnancies in adolescent women. This objective will be achieved using a population-based analysis. An adolescent cohort was assembled using the Missouri linked data set of live births, late fetal and infant deaths from 1978 through 1990.

Project Description

Objectives:

To evaluate the relationship between prior perinatal loss (abortion, fetal and neonatal deaths) and time between successive pregnancies in a cohort of adolescent women.

Methods Employed:

An adolescent cohort of women was assembled from the Missouri linked data set of live births, late fetal and infant deaths from 1978 through 1990. Maternal and infant sibships were organized by a computerized linkage using maternal name, race, age, and parity. Higher-order births identified were linked to the previous birth by year/month of last birth/fetal death. Seven adolescent cohorts' ages <13-19 years were organized based on age at first pregnancy. Kaplan-Meier survival analysis was used for each age cohort as a function of interpregnancy interval. Age cohorts were compared with regard to time to subsequent pregnancy for up to three pregnancy events. Statistical significance of survival curve comparisons between each age cohort and a 24-29 year old reference group was based on the log rank chi square test. Cox regression analysis was employed to compare the hazards based on the outcome of the previous pregnancy controlling for race, maternal education, marital status, maternal smoking, pregravid body mass index (BMI), maternal medical risk factors, and prenatal care utilization. Interpregnancy interval was defined as the time from the previous birth to the next conception (defined by last menstrual period).

Major Findings:

Preliminary results show:

1) The likelihood of a second pregnancy within one year of the first was greater for the youngest adolescents (up to 12%) compared to the reference group.

2) Women who began reproduction as adolescents were more likely to have higher parity (40-65% have at least three pregnancies) compared to the reference group.

3) The rates of abortion were greater for adolescents (30-50%) compared to the reference group (9-11%).

4) The risk of a subsequent pregnancy was 2.5 fold higher in adolescent women with a previous pregnancy loss compared to women with a previous term live birth.

5) Adolescent women tend to have shortened interpregnancy intervals (<1.5 years).

6) Among adolescent women, a prior pregnancy loss was associated with up to a twofold risk of a subsequent pregnancy within 1.5 years compared to women with a previous term live birth.

Significance to Biomedical Research and the Program of the Institute:

This study will enhance our understanding of the phenomenon of reproductive compensation in adolescent women as a factor contributing to shortened spacing between successive births. This information will have a significant public health impact with regard to counseling adolescent women about proper birth spacing to decrease the risk of adverse pregnancy outcomes in this high risk population.

Proposed Course of Project:

The final analysis for this project has been completed.

Publications:

The manuscript is currently under preparation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02522-02 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Intrapartum Antibiotic Prophylaxis against Early-Onset Grp.B Streptococcal Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD
Others:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
	R.A. Brenner	Senior Staff Fellow	EB, DESPR, NICHD
	F.-Y. Lin	Research Medical Officer	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
University of Alabama (B.M. Gray, J.B. Philips); Baylor College of Medicine (L.E. Weisman); Columbia Univ. (J.A. Regan); Univ. of Florida (P. Clark); Univ. Med. Dent. NJ (G.G. Rhoads); Med. Ctr. No. Calif. (P.H. Azimi)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Neonatal group B streptococcal (GBS) disease is the most common disease affecting newborn infants with an estimated incidence of 1.8 per 1000 live births. Twenty-five percent of neonatal GBS disease occurs in premature infants. GBS disease results in significant mortality and morbidity in newborns. Colonization of the infant occurs via vertical transmission from a colonized mother. Among pregnant women, the rate of GBS colonization is approximately 20-30%. Approximately 42-72% of infants born to colonized mothers will become colonized, and 1-2% of these infants will develop invasive GBS disease. A major factor which may modify the rate of colonization, and possibly the risk of invasive disease is intrapartum antibiotic prophylaxis given to women who are at definably high risk for early-onset GBS disease. This intervention strategy has been shown to be near absolute in efficacy trials in conferring protection; it seems probable that substantially lower levels of protection occur when recommended regimens are attempted in routine clinical practice. Supporting this notion is data indicating that intrapartum antibiotic prophylaxis fails to prevent up to 25-30% of cases of early-onset neonatal GBS disease. The purpose of this retrospective case-control study is to evaluate the effectiveness of intrapartum antibiotic prophylaxis given to high risk women in routine clinical obstetric practice with the intent to prevent early-onset neonatal GBS disease. This objective will be accomplished by the following: 1) assembled case and control infants will comprise all live births born to high risk women at the participating hospitals over three years from January 1, 1992 through December 31, 1994; 2) cases and controls will be assembled from a retrospective review of hospital laboratory logs; and 3) perform detailed chart abstraction of clinical and demographic data from the medical records of all eligible case-control maternal and infant pairs.

Project Description

Objectives:

To assess the protection associated with the administration of antibiotics given during labor to high risk pregnant women as prophylaxis to prevent early-onset neonatal group B streptococcal disease (GBS).

Methods Employed:

This is a retrospective case-control study designed to evaluate the effectiveness of intraprtum antibiotic prophylaxis given to high risk women in routine clinical obstetric practice with the intent to prevent early-onset neonatal GBS disease. Cases and controls will be assembled via retrospective review of hospital laboratory logs, hospital discharge diagnoses, and obstetric delivery logs. The sampling frame will comprise all live births occurring at the participating study sites between January 1, 1992 through December 31, 1994; infants born to high risk mothers will be potentially eligible for enrollment in the study. Case infants will be defined as those with documented early-onset GBS disease, and control infants will be defined as those with no documented early-onset GBS disease. Two Controls will be matched to each case based on the hospital of birth, date of birth within three months, and gestational age within two weeks. After assessment of eligibility for enrollment in the study, detailed chart abstraction of clinical and demographic data from the medical records of case and control maternal-infant pairs will take place. Chart abstraction forms designed for this study will be completed on all eligible case and control infants at each study site. These data will be sent to NICHD for data entry, data editing, and analysis.

Major Findings:

None to date (project is under development).

Significance to Biomedical Research and the Program of the Institute:

This study will greatly enhance our understanding of the effectiveness of intrapartum antibiotic prophylaxis intended to prevent early-onset GBS disease in newborn infants. Results from this study will allow better recommendations to be made regarding treatment of mothers and their newborns. In addition, this study of the effectiveness of intrapartum antibiotic prophylaxis will greatly assist in the proper interpretation of the protection conferred by maternal serum antibodies against GBS disease, an NIH sponsored study currently taking place at seven participating U.S. academic centers.

Proposed Course:

The development of a manual of operations and data abstraction forms have been completed. We plan to implement this project beginning July 1996.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HD 02523-02 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies Based on Maternally Linked Birth Registries of Missouri and Utah

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A.A. Herman	Visiting Scientist	EB, DESPR, NICHD
Others:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*

NIDCD (H. Hoffman); Missouri Department of Health (G. Land, J. Stockbauer, W. Schramm); University of Utah (R. Ward, A. Fraser).

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies focus on: 1) the impact of adolescent age at first pregnancy on later pregnancy outcome; 2) the relation between interpregnancy interval and later pregnancy outcomes; 3) the risk of repeating poor pregnancy outcomes. The data sets are unique in that all pregnancies of women from Missouri and Utah are linked into sibships. These data allow the analyst to examine longitudinal pregnancy data.

DESPR developed and cosponsored an international conference on nationally linked data sets. At this conference 6 abstracts were presented: the effect the accumulation of risks on pregnancy outcome, the effect of changing paternity on IUGR, recurrence risks for SIDS, recurrence risks for birth defects and a description of linkage methodologies in the area of maternally linked data sets. A special issue of the journal, Pediatric and Perinatal Epidemiology, is being prepared under the editorial direction of Dr. Herman and two colleagues from CDC & P, with the organizational and financial support of the Division of Epidemiology, Statistics and Prevention Research and its director, Dr. Berendes. Five pages from the two registries will be a part of the special issue.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

1) To examine the impact of adolescent age at first pregnancy and age greater that 35 years at first pregnancy on later pregnancy outcome.

2) The relation between interpregnancy interval and later pregnancy outcomes.

3) The risk of repeating poor pregnancy outcomes. The data sets are unique in that all pregnancies of women from Missouri and Utah are linked into sibships.

Methods Employed:

Analytic methods include risk-based analysis of recurrent mortality and morbidity, life-table analyses and cox regression analysis, indirect standardization of mortality rates by birth weight and gestational age, examination of bivariate distributions using contour diagrams, fuzzy clustering of growth retarded and non growth retarded infants, non-parametric logistic regression, and other procedures applicable to population-based data. The epidemiologic approach is that of a "prospective" cohort design, which permits analyses by case-control, cross-sectional or longitudinal means.

Major Findings:

Abstracts dealing with interpregnancy interval among adolescents (Society for Pediatric Research and Society for Pediatric Epidemiologic Research), increasing risk for perinatal mortality, preterm birth and intrauterine growth retardation among adolescents (Society for Pediatric Epidemiologic Research), sociodemographic profiles and adverse reproductive outcome (American Public Health Association), the recurrence of birth defects (American Society of Human Genetics), and growth retardation and SIDS have been presented in 1995. These results showed that: 1) adolescent women tend to have shorter interpregnancy intervals, 2) the shorter intervals have an adverse effect on pregnancy outcomes, 3) adolescents become pregnant more frequently than older women, 4) risks for perinatal mortality, preterm birth and growth retardation increases with parity among teenagers, 5) adolescents at increased risk for these outcomes have higher rates of poor pregnancy outcome than older women with comparable risk factors, 6) siblings of SIDS infants were not at increased risk for IUGR when maternal smoking was accounted for, 7) both similar and dissimilar birth defects recur with a statistically significant increased frequency, but context and paternity do not further increase the risk, and 8) paternal low SES had a greater influence on low birth weight than maternal low SES. The impact of low SES could not be explained solely by the frequency of high risk behavior among low income families.

Significance to Biomedical Research and the Program of the Institute:

These studies will allow researchers to more fully explore the longitudinal impact of risk factors on pregnancy outcome. The repeater studies are among the first using United States data. Early interventions among high risk women may eventually lead to a reduction of preterm birth and IUGR.

Proposed Course:

The initial year of data analysis has been fruitful. Dr. Herman will continue to examine the longitudinal impact of risk factors, recurrence risk, the impact of low SES on pregnancy and the impact of early - or late first pregnancy on SES.

Publications:

Herman AA, McCarthy B, Bakewell J, Ward R, Mueller BA, Maconochie N, Read A, Zadka P, Skjaevveu R. Data linkage methods used in maternally-linked birth and infant death surveillance data sets from the United States (Georgia, Missouri, Utah & Washington State). Israel, Norway, Scotland and western Australia. Paediatr Perinat Epidemiol (in press).

Herman AA, Yu KF. Adolescent age at first pregnancy and subsequent obesity. Paediatr Perinat Epidemiol (in press).

Krulewitch CJ, Herman AA, Yu KF, Johnson YR. Does changing paternity contribute to the risk of intrauterine growth retardation. Paediatr Perinat Epidemiol (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02524-02 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Occupational Nonfatal Injuries in U.S. Children

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD
Other: K.A. Dunn	IRTA Fellow	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*

U.S. Bureau of the Census (E. Lamas)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.05	.05	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As many as 300 children a year may be killed while working in the U.S.; an unknown number suffer nonfatal injuries on the job. Current information on occupational injuries in children is based entirely on death certificates or workmen's compensation reporting. Both sources are known to contain extensive and biased underreporting for children. Many children are working illegally, "off-the-books", or in hazardous conditions including, but not limited to, those inappropriate for the child's health and developmental status. Determination of high risk occupations and incidence and severity of non-fatal injuries has been made a priority in the injury control and pediatric emergency treatment communities. Current policy questions on effects of 'school-to-work' programs require supporting baseline data on current work practices and injuries. NICHD staff are working collaboratively across Federal Agencies to determine the incidence of non-fatal injuries occurring to children in the workplace by occupational category exposure time. Dr. Dunn is completing a study of occupational exposure time for adolescents ages 15-18 from the Census Bureau Survey of Income and Program Productivity (SIPP). Her work will be used to provide unique and relatively unbiased exposure data for future epidemiological studies. Dr. Overpeck prepared injury questions to be fielded in the SIPP in 1995 and later years. This will enable researchers to target interventions effectively by identifying both high risk children and high risk occupational categories.

PHS 6040 (Rev. 5/92)

Project Description:

Research on adolescent occupational injury in the U.S. has been planned in three phases using the Survey of Income and Program Participation (SIPP). Exposure data for working adolescents is under development now by Dr. Kathleen Dunn. Questions on injuries to adolescents have been added to the SIPP for the 1995 survey and may be included in a new survey under development. The goal is to determine injury incidence and enable researchers to target interventions effectively by identifying both high risk children and high risk occupational categories.

Methods Employed:

The (SIPP) is a continuous household survey fielded by the U.S. Census Bureau. A household is followed for 2 ½ years. Core questions and special topical supplements are administered at four month intervals. SIPP currently determines occupational status, category and time for all adults ≥15 years and tracks those who move. Limited injury questions have been included in the past. Dr. Overpeck has developed questions on injuries at ages 12-17 that have been included for a four-month period of 1995 with a Child Well-being Module sponsored by NICHD's Demographic and Behavioral Science Branch. She may have occupational injury questions included for 50,000 households in the survey to begin in 1996. Injuries will be assessed for medical treatment and severity. Analysis through multivariate regression techniques appropriate for longitudinal complex multistage surveys will be performed.

Major Findings:

Hours worked per week increased with age, with 15 year olds working 14 to 23 hours/week compared to 28 to 33 hours/week for 18 year olds. Occupations varied by age but overall the top two industries were eating and drinking places and grocery stores, and the most common occupation was cashier. During the school year, many young teens are working more hours per week than allowed by child labor laws. These finds have implications for developmental, educational and occupational safety interventions.

Significance to Biomedical Research and the Program of the Institute:

As many as 300 children a year may be killed while working; an unknown number suffer nonfatal injuries on the job. Current information on occupational injuries in children is based entirely on death certificates or workmen's compensation reporting. Both sources are known to contain extensive and biased underreporting for children. Many children are working illegally, "off-the-books", or in hazardous conditions including, but not limited to, those inappropriate for the child's health and developmental status. Determination of high risk occupations and incidence and severity of non-fatal injuries has been made a priority in the injury control and pediatric emergency treatment communities. NICHD staff are working collaboratively across Federal Agencies to determine the incidence and severity of non-fatal injuries occurring to children in the workplace by occupational category exposure time. The ultimate goal is to enable researchers to target interventions effectively by identifying both high risk children and high risk occupational categories.

Proposed Course:

Dr. Dunn's work will be used as part of her dissertation for a degree in Epidemiology at the University of North Carolina and will be submitted for publication. An abstract has been submitted for the APHA annual meeting.

Dr. Overpeck will use the preliminary work on exposures prepared by Dr. Dunn to evaluate the injury data that will be available for the first time in late 1996.

Publications: None to date

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02525-02 EB

PERIOD COVERED

October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

World Health Organization Study of Health Behavior in School Children (WHO

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD
Others:	N. Krasnegor	Chief, HLBB	HLBB, CRMC, NICHD
	P.C. Scheidt	IPA	HLBB, CRMC, NICHD

COOPERATING UNITS *(if any)*

Substance Abuse and Mental Health Services Admin. (B. Rouse); Bar Ilan University, Israel (Y. Harel); WHO-HBSC Study Group (27 countries)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The WHO coordinates a quadrennial survey that compares health behavior in adolescents in 27 countries. The survey is used to monitor and investigate patterns, determinants and effects of health related behaviors including smoking, substance use, and injury behavior. NICHD staff attended meetings with the international working group to learn about the process of comparing the data and results of the most recent international studies. The U.S. has been provisionally accepted as a participating member subject to successful completion of a survey meeting the tight requirements of the WHO-HBSC steering committee. The Substance Abuse and Mental Health Services Administration (SAMHSA) has funded and fielded a U.S. school-based survey that will meet the preliminary criteria for U.S. participation in the WHO-HBSC project. The successful completion and analysis of the U.S. survey and linkage to the existing data of the ongoing WHO surveys will allow the U.S. to compare behavior and related injury factors to data from 27 countries of Europe, Canada, and elsewhere. The Epidemiology and Human Learning and Behavior Branches of NICHD are collaborating with SAMHSA to conduct the data analysis for both the U.S. and international data sets. NICHD will focus particularly on injury comparisons. The data will prove invaluable for both international and domestic comparisons of injury and related behaviors in adolescents to target interventions since injury is the leading cause of death for children in the U.S.

Project Description:

Objectives:

The World Health Organization Study of Health Behavior in School Children (WHO-HBSC)is used to monitor and investigate patterns, determinants and effects of health related behaviors including smoking, substance use, and injury behavior. The U.S. has not been a participant. Collaboration with the Substance Abuse and Mental Health Services Administration (SAMHSA) has produced the survey data. NICHD will focus particularly on injury and related behaviors.

Methods Employed:

The WHO coordinates a quadrennial survey that compares health behavior in adolescents in 27 countries. SAMHSA collected comparable data in a school-based study of U.S. adolescents during 1996. NICHD collaborators in the intramural and extramural programs will use descriptive and multivariate epidemiologic techniques appropriate for complex multistage complex surveys to prepare analytic papers.

Major Findings: None available yet.

Significance to Biomedical Research and the Program of the Institute:

Injury is the leading cause of death for children in the U.S. NICHD has been active in promoting injury research as an emerging new endeavor through behavioral and epidemiological analyses and will emphasize this area in the collaborative analyses. The U.S. has learned much about risk factors and health outcomes through such international comparisons on infant mortality, aging, heart disease and health behaviors, for example. These data will prove invaluable for both international and domestic comparisons of injury and related behaviors in adolescents to target interventions appropriate to the U.S.

Proposed Course:

NICHD has proposed joint funding by the intramural and extramural programs to support analysis of the SAMHSA survey data. The collaborative research team will complete the work through an interagency agreement with SAMHSA which has an ongoing contract to perform the survey and complete the analysis.

Publications: None to date

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02526-01 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Survival and Health Status Analysis of Premature, High-Risk Deliveries

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
National Perinatal Information Center (J. Muri, R. Schwartz, J. Pezzullo);
National Center for Health Statistics (M. Kogan, J. Weed)

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.2	.2	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This national study will determine the effect of the available level of technology at the hospital of delivery on survival and subsequent health status of children at age three. The national study incorporates oversamples of very low birth weight infants as well as fetal and infant deaths. Survival time of deliveries will be analyzed by age at death for infants or gestational age for a fetal death, symptoms at presentation, use of interventions, and level of technological support available at the delivery hospital while accounting for gestational age and other risk factors. Health status differences at age three will be obtained from measures included in a 1991 longitudinal follow-up of a nationally representative survey of all children born in 1988 performed by the National Center for Health Statistics (NCHS). The health outcome measures will include development, overall health status, chronic conditions, recent illness, use of apnea monitors and ear tubes, vitamin/mineral use, and use of home treatments. Hospital and physician records from the perinatal and pediatric period are included in the analysis.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

This nationally representative study will assess the effect of the available level of technology at the hospital of delivery on survival and subsequent health status of high risk children at age three.

Methods Employed:

Data for the study have been linked from multiple sources: (1) the confidential NCHS files of the 1988 National Maternal and Infant Health Survey (NMIHS); (2) the Longitudinal Follow-up to the NMIHS (LF-NMIHS) and (3) the American Hospital Association (AHA) files on the technological capacity of hospitals of delivery for the 1988 birth cohort supplemented with in-depth descriptions of capacity from a special survey of delivery hospitals performed by the National Perinatal Information Center (NPIC). The LF-NMIHS included all women whose child born in 1988 was alive in 1991 and samples from the fetal and infant deaths identified previously in the NMIHS. The NMIHS files include the identification of delivery hospitals which will be used to link information across the LF and the AHA data. The delivery hospitals are supplying information on use of experimental surfactant therapy protocols during 1988 when deliveries occurred.

Major Findings:

None yet.

Significance to Biomedical Research and the Program of the Institute:

This nationally representative survey will provide baseline data on outcomes of deliveries occurring before surfactant therapy was approved for use by FDA. Clinical follow-up studies of health outcomes of high risk births will be able to use this study as reference data for current therapies including surfactant and antenatal corticosteroid therapy. Changes in delivery outcomes from fetal death to live birth and subsequent survival will be used to document changes occurring in the high risk infant cohorts due to advanced techniques of care.

Proposed Course:

The National Perinatal Information Center has successfully linked the confidential data of the NMIHS to the AHA data files. Data verification is under way. Several analyses involving regression and survival models are proposed for publication.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02527-01 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Fetal Growth in Chronically Malnourished Women

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD
Others:	N. Tafari	Visiting Scientist	EB, DESPR, NICHD
	J.D. Clemens	Chief	EB, DESPR, NICHD
	M. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
Z.W. Gabriel Director, Ethiopian Nutrition Institute

LAB/BRANCH
Epidemiology Branch

SECTION

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This study was undertaken in a population of chronically malnourished women of Central Ethiopia (Addis Ababa) from June 1987 through February 1988. The purpose of the study was to determine the impact of severe food energy deprivation on fetal growth. We also intended to determine whether the level of maternal food energy intake was associated with a reduction of maternal body nutritional stores as measured by triceps skinfold thickness and body mass index (BMI).

Women were recruited for this study during their first prenatal visit to the primary health care clinic. Women were interviewed consecutively, 136 of the 457 women interviewed were eligible for enrollment. The enrollment criteria consisted of known gestational age, stable family composition, pregnancy less than 32 weeks' gestation, and informed consent to participate in a four-day home diet survey. In addition, women consented to have anthropometric measures performed during each of the three prenatal visits and two postpartum visits. Outcome measures included birth weight, maternal BMI, food energy intake as a multiple of basal metabolic rate, observed caloric intake, and maternal triceps skinfold thickness.

The important results of this analysis were that low birth weight (<2500 grams) were not an inevitable consequence of chronic maternal malnutrition. We also found that maternal food energy intake was highly correlated with a reduction in maternal body nutritional stores.

PHS 6040 (Rev. 5/92)

Project description:

Objectives:

To evaluate the impact of chronic maternal malnutrition on fetal growth. In addition, to determine if the level of maternal food energy intake was associated with a reduction of maternal body nutritional stores as measured by triceps skinfold thickness.

Methods Employed:

Subjects for this study were enrolled during their first prenatal visit to the primary health care clinic. Each woman was interviewed, and enrollment criteria were assessed. The enrollment criteria included known gestational age as determined by LMP, stable family composition, pregnancy less than 32 weeks' gestation, and informed consent to a four-day home diet survey, as well as anthropometric measures at each of the three prenatal visits and two postpartum visits. These measurements included triceps skinfold thickness, subscapular and suprailiac skinfold thickness, and midarm circumference. In addition, maternal weight, height, blood pressure, and hematocrit were obtained during the prenatal and postnatal visits. The home diet survey consisted of measurement of energy, protein, and other nutrient content of the home diet. These were estimated over four consecutive days (Tuesday through Friday) via weighted inventory survey. The survey team noted the individual food items planned by the cook. The prepared meal was weighed before and after consumption. Differences in these measures were considered the daily food consumption by that household.

Major Findings:

1) The women in this study population were found to be severely malnourished with food energy consumption 6% above basal metabolic requirements.

2) The level of maternal food energy intake was associated with a reduction of maternal body nutritional stores as measured by triceps skinfold thickness and BMI.

3) Low birth weight births are not an inevitable consequence of chronic maternal malnutrition. Infants born to women in this study population were normally grown. The mean birth weight, length, and head circumference were well within the 50th percentile.

Significance to Biomedical Research and the Program of the Institute:

The results of this study profoundly impact the assumptions frequently made with regard to our knowledge of the effects of maternal malnutrition on fetal growth. These findings suggest that an effective compensatory physiologic mechanisms play an important role in protecting the developing fetus from severe growth restriction as a result of maternal malnutrition. These findings may provide better data to evaluate the effectiveness of food supplementation programs, and determine the most appropriate target populations for these programs.

Proposed Course:

The analysis has recently been completed. A draft manuscript has been developed.

Publications: (None) Manuscript in preparation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02528-01 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Epidemiology of Typhoid Fever in India

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.D. Clemens	Chief, EB	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD
	A. Naficy	Expert	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
AIIMS, Delhi (M.K. Bhan, S. Sazawal, R. Kumar);
TRC, Madras (M. Datta).

LAB/BRANCH
Epidemiology Branch

SECTION
Pediatric Infectious Diseases and Vaccinology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Typhoid fever is perceived to be a major problem in India, but its true incidence is uncertain. In preparation for future field trials of typhoid vaccines, this research is establishing two field sites, an urban site in Delhi and a rural site in Chingleput, near Madras, to monitor typhoid incidence in cohorts of subjects. The site in Delhi comprises a population of about 19,000 persons, while that in Chingleput has nearly 33,000 persons. Six months of surveillance undertaken between November 1995 and May 1996 in Delhi projects an annual incidence of up to 4.6 cases per 1,000, while 3 months of surveillance, begun in March 1996 in Chingleput, projects an incidence of 1 per 1,000. All cases of typhoid are being followed for up to 3 months to determine typhoid-related disability, and case-control studies are nested within these cohorts to assess foods, water sources, and hygienic practices associated with typhoid risk, as well as the role of H. pylori coinfection in influencing this risk. Future serosurveys will enable determination of the age-specific seroprevalence of anti-d antibodies, to provide complementary evidence addressing the burden of typhoid.

Project Description:

Personnel: Dr. John Clemens of the Epidemiology Branch, DESPR, is the principal investigator of this project, with responsibility for the epidemiological design and analysis of the study. Dr. Abdollah Naficy and Mr. Malla Rao of the Epidemiology Branch are providing support for these activities. Execution of the project in Delhi is under the direction of Professor M.K. Bhan of the All India Institute of Medical Sciences; direction in Chingleput is being provided by Dr. M. Datta of the Tuberculosis Research Centre (TRC) of Madras.

Objectives:

1. To estimate the age-specific incidence of typhoid fever in two field sites, one urban (Delhi) and one rural (Chingleput).

2. To evaluate whether the detected incidence of typhoid fever is augmented by active (household) surveillance.

3. To determine hygienic and dietary risk factors for typhoid fever in these field sites.

4. To assess whether infection by Helicobacter pylori is a risk factor for typhoid fever in these field sites.

5. To measure the duration and extent of disability associated with typhoid fever.

6. To measure the age-specific seroprevalence of serum anti-d antibodies in these two populations.

Methods Employed:

The study assembles the entire population of each field site for longitudinal follow-up to detect typhoid fever. Within each population, random samples of about 8,000 persons are visited in their households twice-weekly to detect cases of febrile illness, while surveillance of the remainder comprises monitoring of cases presenting to established treatment settings. Typhoid fever is diagnosed by collection of blood cultures. Disability associated with typhoid is assessed by administering a questionnaire to cases of typhoid, weekly up to three months after diagnosis. Risk factors for typhoid are assessed by interviewing typhoid cases and non-typhoid controls; serum anti-H. pylori antibodies, assessed in blood collected from cases and controls, are used to evaluate whether infection by this organism predisposes to typhoid fever. Blood will be collected from random samples of subjects selected from each community to measure anti-d antibodies.

Major findings:

The initial 6 months of surveillance undertaken between November 1995 and May 1996 in Delhi projects an annual incidence of up to 4.6 cases per 1,000, while the initial 3 months of surveillance, begun in March 1996 in Chingleput, projects an incidence of 1 per 1,000.

Significance to Biomedical Research and the Program of the Institute:

Typhoid is a major problem worldwide, and development of new typhoid vaccines has been accorded major priority by NICHD. Development of these two field sites will provide opportunities to test the clinical efficacy of new typhoid vaccines developed at NICHD and elsewhere.

Proposed Course:

This is envisioned as a two-year project (project began in November 1995).

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02529-01 EB

PERIOD COVERED
October 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Field Trial of Killed Oral Cholera Vaccines in Vietnam

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD
	A. Naficy	Expert	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
National Institute of Hygiene and Epidemiology, Hanoi (D.D. Trach);
University of Goteborg (J. Holmgren);
University of Maryland (A. Sorkin, B. Stanton).

LAB/BRANCH
Epidemiology Branch

SECTION
Pediatric Infectious Diseases and Vaccinology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.8	1.8	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cholera remains a significant public health problem in Vietnam. During the late 1980s, the National Institute of Hygiene and Epidemiology of Vietnam developed a killed oral cholera vaccine modelled on a Swedish vaccine tested by Branch investigators in Bangladesh during the mid-1980s. A field trial of this vaccine in Hue, which enrolled 100,000 subjects, found the vaccine to confer about 60% protection against El Tor cholera, with similar efficacy in young children and in older persons. This earlier vaccine has now been augmented to include coverage against the newly recognized 0139 serogroup of V. cholerae. A pretest of the safety and immunogenicity of this vaccine was recently conducted in adults in Hanoi. During the next 9 months, a similar pretest will be conducted in Hanoi children, and a large-scale field trial of the vaccine will be initiated in the coastal city of Nha Trang next March. This trial will enroll about 200,000 persons, and will assess the clinical effectiveness of the vaccine over two years following vaccination.

PHS 6040 (Rev. 5/92)

Project Description:

Personnel: Dr. John Clemens of the Epidemiology Branch, DESPR, is the principal investigator of this project, with responsibility for epidemiological design and analysis of the study. Dr. Abdollah Naficy and Mr. M.R. Rao of the Branch are assisting in these activities. Implementation of the study is under the direction of Prof. D.D. Trach, Deputy Director of the National Institute of Hygiene and Epidemiology in Hanoi. Laboratory support is being provided by Prof. Jan Holmgren of the University of Goteborg.

Objectives:

1. To assess the safety and immunogenicity of a bivalent (01-0139), killed oral cholera vaccine in adults and children in Vietnam.

2. To measure the clinical effectiveness of this vaccine in preventing cholera in central coastal Vietnam.

Methods Employed:

In the two pretests, subjects are randomized to vaccine or placebo; symptoms are measured over a two-week period after each dose; and bloods, obtained at baseline and two weeks after the second dose, are assessed for serum vibriocidal antibody responses to the vaccine. In the main trial, approximately 200,000 persons residing in the Nha Trang and Ninh Hoa districts of Khanh Hoa Province will be randomized to vaccine or to placebo. Each agent will be given in a two-dose regimen. Vaccine safety will be assessed by monitoring visits to Nha Trang and Ninh Hoa hospitals. Vaccine protection will be measured by conducting surveillance for all visits for treatment of diarrhea in government health facilities, and by arranging for fecal culturing of these diarrheal patients for V. cholerae.

Major Findings: None to date.

Significance to Biomedical Research and Program of the Institute:

Cholera is a major public health threat, particularly in developing countries of Asia, Africa, and Latin America. Recently developed cholera vaccines, however, are too expensive to be deployed for the control of cholera in these settings. A killed, oral cholera vaccine developed in Vietnam, which costs only pennies per dose, may be a useful cholera vaccine that is within reach of the limited health care budgets of these countries. Before recommending that such a vaccine be used in these settings, however, it is essential that the vaccine be evaluated in a rigorous, randomized, double-blind vaccine trial. The present research constitutes such a trial. If found to be suitably safe and effective in this trial, this vaccine may represent an important public health tool for developing countries.

Proposed Course: This research is envisioned as a four-year project.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD02530-01 EB

PERIOD COVERED
December 1, 1995, to September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Economic Analysis of Killed Oral Cholera Vaccine in Refugee Settings in Malawi

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A.B. Naficy	Expert	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief, EB	EB, DESPR, NICHD
	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS *(if any)*
University of Maryland Baltimore County, Baltimore (A. Sorkin)
Epicentre, Paris (C. Paquet)

LAB/BRANCH
Epidemiology Branch

SECTION
Pediatric Infectious Diseases and Vaccinology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ © Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Currently, the provision of an adequate quantity of purified water, the chlorination of contaminated water sources, and the establishment of isolated areas for defecation pending appropriate sanitation systems, are measures taken to help prevent cholera outbreaks in refugee settings. Beyond prevention, the mainstay of cholera control remains appropriate case management to prevent mortality. The current approach taken is to provide the necessary supplies and equipment to manage cases after the epidemic has been recognized, an approach we define as reactive treatment. The recent availability of oral cholera vaccines, and the demonstration in large scale field trials that the inactivated whole-cell and B subunit vaccine is both safe and efficacious, has renewed interest in cholera vaccination as a potential public health measure to prevent cholera outbreaks in situations with high cholera incidence, such as refugee populations. In view of limited available resources, to assist in formulating policy decisions regarding appropriate public health measures to control cholera outbreaks in refugee situations, we are preparing a cost-effectiveness analysis of several alternative intervention strategies that incorporate case treatment and/or vaccination against cholera. The results to date indicate that in the absence of a vaccination intervention to control cholera in refugee settings, a preemptive treatment strategy (setting up the infrastructure and providing the supplies required to manage a cholera outbreak at inception of the camp) is expected to cost less and save more lives than the current reactive treatment strategy. If vaccination is to be incorporated into a treatment strategy, intervention strategies other than the combination of preemptive vaccination (vaccination of the whole refugee camp at inception of the camp) and preemptive treatment prevent no further cholera cases, prevent fewer cholera deaths, and cost more. The final analyses with precise estimates and the results of the sensitivity analyses should be available by September 1996, when the project is envisioned to end.

Project Description:

Personnel:

Dr. Abdollah Naficy of the Epidemiology Branch, DESPR, is the principal investigator of this project, with responsibility for the design and analysis of the study. Dr. John Clemens and Mr. Malla Rao of the Epidemiology Branch, DESPR, and Dr. Christophe Paquet of Epicentre, Paris, are providing support for these activities. Dr. Alan Sorkin of the University of Maryland Baltimore County, Baltimore, is providing additional support in the economic aspects of the study.

Objectives:

1) To estimate the health benefits and the costs of intervention strategies to prevent and control cholera outbreaks in refugee settings.

2) To estimate the most cost-effective intervention strategy to prevent and control cholera outbreaks in refugee settings.

3) To identify parameters to which the cost-effectiveness analysis is most sensitive.

Methods Employed:

This analysis addresses the cost-effectiveness of several alternative interventions for preventing and managing cholera outbreaks in refugee settings. Based on consideration of realistic intervention strategies that might be undertaken in refugee settings, the following candidate intervention strategies have been selected for comparison in the cost-effectiveness analysis:

1) Preemptive treatment alone.

2) Preemptive vaccination combined with preemptive treatment.

3) Preemptive vaccination combined with reactive treatment.

4) Reactive treatment alone.

5) Reactive vaccination combined with reactive treatment.

We define preemptive vaccination as vaccination of the whole refugee camp at inception of the camp, and reactive vaccination as vaccination of the refugee camp after the outbreak of cholera has been recognized. Preemptive treatment is defined as setting up the infrastructure and providing the supplies required to manage a cholera outbreak at inception of the camp, as opposed to reactive treatment, in which the necessary supplies and equipment are provided after the epidemic has been recognized. All intervention strategies have been compared to one another, and also to a baseline standard of care of no supplementary intervention to prevent or control cholera. The final indices of intervention performance are expressed as cost per case prevented and cost per death averted. The setting chosen for the analysis is a hypothetical refugee camp with an overall population of approximately 50,000 refugees, situated in a cholera endemic area of sub-Saharan Africa. The vaccine chosen for the analysis is the killed oral whole-cell and B subunit (WC/BS) vaccine, since it is the only oral cholera vaccine which has completed large scale field trials in endemic settings. Medecins Sans Frontieres (MSF), and Epicentre (an expert group of practical epidemiologists), between 1987 and 1993, have collected detailed epidemiologic and cost related information on cholera outbreaks in Mozambican refugee camps in Malawi. This data has served to increase the precision of estimates incorporated into our base-case analysis. Remaining parameter estimates have been obtained from experts in the field or from the published literature. We have used sensitivity analyses to explore the impact of varying these best estimates throughout plausible ranges. To estimate costs, we have decided to incorporate all medical costs entailed in vaccination and treatment. Only direct costs are included in the model; this excludes such hypothetical costs as the monetary value of human life. The model is based on local costs for supplies and personnel that are obtained from 1995 costs in Malawi; external costs are based on actual costs incurred by MSF.

Major Findings:

In the absence of a vaccination intervention to control cholera in refugee settings, the base case analysis demonstrates that a preemptive treatment strategy is expected to cost less and save more lives than a reactive treatment strategy. If vaccination is to be incorporated into a treatment strategy, intervention strategies other than the combination of preemptive vaccination and preemptive treatment prevent no further cholera cases, prevent fewer cholera deaths, and cost more.

Significance to Biomedical Research and the Program of the Institute:

With the rapidly growing number of refugees worldwide, cholera outbreaks in such populations which result in significant morbidity and mortality have become an increasingly recognized public health problem; both in children and in adults. The availability of different approaches to case management, the availability of safe and efficacious oral cholera vaccines, and the limited resources available in developing countries where most of these cholera outbreaks occur, makes it necessary to estimate the cost-effectiveness of different intervention strategies that can be incorporated as public health measures to prevent and control cholera outbreaks in such refugee populations.

Proposed Course:

The project is envisioned to end in September 1996.

Publications:

None.

Project Numbers - Annual Report 1996

PREVENTION RESEARCH BRANCH (PRB), DESPR

ZO1 HD 00876-06	Determinants of Childhood Poison Ingestion	Lois A. Maiman, Ph.D.
ZO1 HD 00877-05 **TERMINATED**	Prevention and Education in Coronary Heart Disease Reduction	Lois A. Maiman, Ph.D.
ZO1 HD 2100-04 **TERMINATED**	Prenatal Care Utilization and Adverse Health Behaviors During Pregnancy	Lois A. Maiman, Ph.D.
ZO1 HD 2101-05	Increasing Preventive Actions for Childhood Injury Among Preschool Children	Lois A. Maiman, Ph.D.
ZO1 HD 2110-05	Prevention of Problem Behaviors Among Middle School Students	Bruce G. Simons-Morton, Ed.D., M.P.H.
Z01 HD 2114-04	MD/Family Partnership: Education in Asthma Self-Management	Lois A. Maiman, Ph.D.
Z01 HD 2115-04	Immunization Among D.C. Infants	Bruce G. Simons-Morton, Ed.D., M.P.H.
Z01 HD 2116-04	Preventing Pregnancies Among Adolescent Girls in the District of Columbia	Bruce G. Simons-Morton, Ed.D., M.P.H.
Z01 HD 2119-03 **PRJT. COMPLETED (10/1-12/31/95)**	Parenting Style and Adolescent Substance Use and Depressive Symptoms	Barbara Radziszewska, Ph.D.
Z01 HD 2120-02	A Survey of Parent Intervention Strategies to Prevent Adolescent Alcohol Use	Bruce G. Simons-Morton, Ed.D., M.P.H.
Z01 HD 2121-02	Parent-Child Interaction and Asthma Self-Management	Lois A. Maiman, Ph.D.
Z01 HD 2122-02	Perceptions of Parents Regarding the Onset of Sexual Intercourse Among Youth	Aria D. Crump, Sc.D.
Z01 HD 2123-01 **(4/1-6/30/96)**	Role of Parental Guidance in Pediatric Asthma Self-Management in Inner-City Population	Barbara Radziszewska, Ph.D.

ZO1 HD 2124-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00876-06 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Determinants of Childhood Poison Ingestion

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
	P. Moyer	Computer Specialist	CSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Medicine and Dentistry, University of Rochester (N.G. Hildreth, R.A. Lawrence, G.S. Liptak)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.225	0.20	0.025

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Determinants of Childhood Poison Ingestion study investigates the relationship between social and behavioral factors and risk of childhood ingestions of household cleaning and home repair and maintenance products.

Project Description

Objectives:

The specific aims of the study are: 1) to determine the sociodemographic, predisposing, life events and situation and social network factors, parent health protective behaviors for children, and household hazards that affect childhood poison ingestion; and 2) to examine the relative contributions of these factors to poison ingestion. Funding for this project was provided by the HBL, CRMC, NICHD to the Principal Investigator at the time she was Associate Professor, Department of Pediatrics, University of Rochester. Specifically, the PRB, DESPR is responsible for all analysis and dissemination of study findings.

Methods Employed:

Using a case-control design, a proportionate systematic random sample of cases (10 months to 5 years of age; N = 326) were selected from calls for assistance to Poison Control or visits to pediatric emergency department for ingestions of household cleaning or home repair and maintenance products. Controls matched on age of child, N = 326) were obtained from community pediatric practices (N = 15). The primary caretaker responsible for the health of the child was interviewed within two weeks of the ingestion or control selection.

Adjusted analyses for separate models of major study dimensions employed multiple logistic regression. The presence of covariates yielded nonsignificant demographic effects (mother's age, education and occupational prestige, family income, and marital status; child's gender) with the exception that mothers working full-time were less likely than those not employed to have a case child (OR = .49, 95% CI = 0.32, 0.74). There were no case-control differences associated with ingestions of other substances or number of other types of injuries, where as regular daytime care in the child's own home compared to a day care center produced a significant effect (2.15, 1.02, 4.51). There was an association between reports of a greater number of major frequent life events and being a case (OR = 1.38, 95% CI = 1.11, 1.70) as well as having living fewer months in the family's current home. The presence of a salient life event was more likely to be reported by mothers of controls (OR = .64, 95% CI = 0.42, 0.98). When adjusted for measures of social network and network interaction, more interaction with a spouse/partner about the child yielded a protective effect (OR = .28, 95% CI = 0.08, 0.96) and subjects interacting more frequently with relatives other than the spouse or friends about the child were more likely to be a case than a control (OR 1.03, CI = 1.00, 1.06). A model containing measures of child risk behaviors and mother's hazard behaviors showed a significant association for a greater number of child risk behaviors and ingestion (OR = 1.58, 95% CI = 1.15, 2.17).

Significance to Biomedical Research and the Program of the Institute:

The study findings will provide recommendations for intervention strategies to reduce risk factors for ingestion which can be used in subsequent experimental studies to determine their effectiveness and contribution to the field of childhood injury prevention.

Proposed Course:

Manuscript preparation is now in progress.

Publications:

None to date.

Contract:

No contracts will be issued for this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00877-05 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prevention and Education in CHD Reduction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	R. DerSimonian	Mathematical Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Medicine, Northwestern University (P. Greenland) and School of Medicine and Dentistry, University of Rochester (N.G. Hildreth, C. Cox)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.125	0.125	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Prevention and Education in Coronary Heart Disease (CHD) Reduction study investigates issues related to the future design and testing of behavioral and educational strategies to help children and adolescents reduce risk behaviors and acquire and practice positive health behaviors.

THIS PROJECT IS COMPLETED, PLEASE DISCONTINUE

Project Description

Objectives:

The major goal of this research is to identify the unintended functional and psychological consequences of CHD risk identification. The specific aims of the study are: 1) to identify whether risk identification influences labeling effects in terms of decreased physical and role functioning and untoward health perceptions; and 2) to determine whether false reassurance occurs for individuals informed they are not at-risk as operationalized by undertaking poorer life style behaviors (e.g., cigarette smoking, unhealthy diet, sedentary habits) and reduced perceptions of health risks.
Funding for this project was provided by the DHVD, NHLBI to Dr. Maiman at the time she was Associate Professor, Department of Pediatrics, University of Rochester and Co-Principal Investigator on the project. The PRB, DESPR is responsible for analysis and dissemination of study findings from the screening program related to functional, behavioral, and psychological outcomes.

Methods Employed:

7,284 individuals attended public cholesterol screening and 2,109 with elevated levels were referred for retesting and diagnosis. A 2 X 3 factorial design (health professional or lay counselor X incentive offer, reminder letter or control) was employed to examine whether interventions affect compliance with referral. In addition, 3 surveys (baseline, 5 months after screening, and one year after screening) collected information on sociodemographics, risk factors, social support and health perceptions. Cholesterol reassessments were conducted one year after screening. A comparison group of non-elevated subjects was included in the follow-up observations, yielding a data set of 3,352.

Significance to Biomedical Research and the Program of the Institute:

Evidence suggests that behaviors associated with increased CVD risks (cigarette smoking, high fat and high cholesterol diet, sedentary habits) are acquired early in life and may accelerate the development of CVD. Primary prevention programs may prevent CVD, delay the onset and reduce the severity of the disease and associated costs of medical care. However, research is needed to develop strategies to help young people reduce risk behaviors and acquire and practice positive health behaviors. Accompanying such efforts is the need to alleviate possible unintended and negative consequences of such interventions, such as labeling and false reassurance. The information from this project will contribute to the development by the PRB of a program of CVD risk reduction interventions for children and adolescents in which behavioral and educational strategies will be used.

Proposed Course:

Data have been analyzed and manuscripts prepared for submission for publication. Project is completed.

Publications:

None on major questions of interest to PRB available at this time.

Contract:

No contracts will be issued for this study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2100-04 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prenatal Care Utilization and Adverse Health Behaviors During Pregnancy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.025	0.025	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research involves a program of studies employing behavioral and social support interventions to improve early entry to prenatal care and adherence to the visit schedule and decrease adverse health behaviors during pregnancy. The studies will focus on adolescent females who are at high risk of obtaining late or no prenatal care and particularly vulnerable to negative health behaviors (e.g., smoking, alcohol abuse, poor nutritional choices, illicit drug use) that are associated with poor pregnancy and birth outcomes.

THIS PROJECT IS TERMINATED DUE TO INSUFFICIENT RESOURSES, PLEASE DISCONTINUE.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The overall goal of the program of studies is to help prevent negative outcomes associated with adolescent pregnancy.

Methods Employed:

The first study in this series will focus on utilization of prenatal care. A school-based program to increase early entry to prenatal care visits is planned. The next study will build on the efficacy of this intervention to promote cessation of negative health behaviors.

Significance to Biomedical Research and the Program of the Institute:

Healthy People 2000 objectives indicate the need to improve early entry to prenatal care and reduce adverse health behaviors during pregnancy.

Proposed Course:

Project is terminated due to insufficient resources.

Publications:

None.

Contract:

No contracts were issued for this study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2101-05 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Increasing Preventive Actions for Childhood Injury

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major purpose of the Increasing Preventive Actions for Childhood Injury study is to develop and test an intervention to reduce parental unsafe practices for childhood injuries in the home. A multicomponent preventive intervention for pediatric health care providers and parents will be developed and evaluated.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The primary analysis of interest will be to assess the effect of the interventions on level of unsafe practices in home by parents related to childhood injuries. Additional major areas of interest are to determine the effect of the provider intervention on physician advocacy and competency in injury prevention and on parent participation in the follow-up injury prevention program.

Methods Employed:

An experimental design will be employed with physicians assigned to no treatment (control) or injury prevention education intervention. A parent-child sample will be obtained for each pediatrician and randomized to either follow-up injury prevention program or standard treatment. The study will focus on children 10 months to 5 years of age.

Significance to Biomedical Research and the Program of the Institute:

Research on educational and motivational strategies for injury control needs to respond to complex issues including the mechanism of effective approaches and identification of subpopulations who may respond differential to such intervention. Studies have demonstrated the influential roles of physician information, attitudes and behavior on the adoption of recommended practices of patients. It appears appropriate to enhance to the capacity of physicians to take an active role as motivators and advocates for injury prevention and to recognize the social and behavioral factors associated with high risk families.

Proposed Course:

A concept review has been completed and approved by an independent panel of reviewers. The RFC is being written for internal NICHD review and approval.

Publications:

None yet.

Contract:

A contract will be developed for the project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2110-05 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Preventing Problem Behavior Among Middle School Students

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD
Others:	L.A. Maiman	Chief	PRB, DESPR, NICHD
	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
	D. Haynie	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
	A. Crump	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
	K. Yu	Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Behavior and Health Research, Inc. (K. Saylor)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of the study is to develop, implement, and test the efficacy of a multi-component program of school-based interventions in preventing problem behavior in a sample of middle school students. Interventions are directed at the students to improve their resistance to problem behavior and toward the school environment and parent behavior to provide support for student prosocial behavior. The primary research question is, "What is the effect on the frequency of problem behavior among students participating in a special intervention that includes an orientation to middle school, nontraditional curriculum, and parent intervention compared to students in the comparison group?"

Project Description

Objectives:

1. Evaluate the extent and quality of implementation of the intervention components.
2. Evaluate the impact of the intervention on selected mediating variables, including student and parent self efficacy, expectancies, and perceived social norms.
3. Evaluate the impact of the intervention on the extent of student problem behavior.

Methods Employed:

Measurement: Baseline survey of problem behavior and hypothesized mediators; school records; parent survey.
Intervention: Curriculum, school environment, and family education.
Design: Seven schools will be randomized to special intervention or usual care. Two cohorts of 6th graders (n = 2600) will be followed to 9th grade.

Major findings: None yet

Significance to Biomedical Research and the Program of the Institute:

Problem behavior is an important health problem among adolescents. The mission of the PRB is to conduct intervention studies to improve health and change health behavior of children and adolescents. This trial will test the efficacy of a multi-component program on problem behavior. Future studies can test the effectiveness of the components.

Proposed Course:

The interventions have been developed and pilot tested, and the OMB application has been approved. Baseline data collection is scheduled for September, 1996.

Publications:

None

Contract Report:

Behavior and Health Research, Inc.
Contract No. N01-HD-4-3207
Initiation date: June 15, 1994
Title: Preventing Problem Behavior Among Middle School Students
Current funding: $460,896
Objectives: Develop and test multicomponent, school-based interventions to prevent problem behavior.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2114-04 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

MD/Family Partnership: Education in Asthma Self-Management

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	P. Moyer	Computer Specialist	CSB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Public Health, University of Michigan (N.M. Clark, D.W. Roloff, M.E. Hurwitz, M.A. Schork) and School of Medicine, Columbia University (R.B. Mellins, D.

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.25	0.20	0.05

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major purpose of the MD/Family Partnership: Education in Asthma Self-Management study is to develop and test an intervention based on theories of self-regulation of behavior in changing the diagnosis and treatment patterns and educational behavior of physicians who treat children with asthma.

Project Description

Objectives:

It is hypothesized that education for physicians, based on the principle of self-regulation provided through an interactive seminar, will be effective in changing physicians' clinical behavior and improving their performance as health educators and counselors. The study also assesses the effects of education for pediatricians on the self-management behavior, health status, and health care utilization of their patients.

This research builds on an earlier study funded by the Agency for Health Care Policy and Research and NICHD to Dr. Maiman at the time she was a faculty member at the Department of Pediatrics, University of Rochester School of Medicine and Dentistry. Dr. Clark is Principal Investigator on the current study and funding is from NHLBI, NIH.

Methods Employed:

A randomized experimental design involving 60 pediatricians and 800 parent-child pairs from their practices will be employed. Data will be collected in Ann Arbor, Michigan and New York City. The first stage has been devoted to the development of the interactive seminar and video vignettes; development and field testing of physician and parent data collection instruments; and recruitment of study physicians. Baseline information has been collected from 58 physicians and 789 parent-child pairs, and 28 physicians attended the educational seminars. The first wave of follow-up data has been collected from both physicians and parents and the second wave is in progress.

Significance to Biomedical Research and the Program of the Institute:

A strong partnership between physicians and patients has been discussed as a crucial part of effective asthma management. The study is unique in that it is the first evaluation research to focus on pediatricians who treat asthma and to apply theories of self-regulation of behavior to physician education. It is one of few to use changes in patient self-management behavior and health care use as outcomes of physician education. Findings will be important for the design of physician education related to asthma self-management and the strengthening of the partnership.

Proposed Course:

The study will be conducted over a five year period, funded by NHLBI. Manuscripts are in process from baseline and initial follow-up data. The second follow-up assessments began in April 1996 and will be completed in December 1996. Manuscripts will be written based on these data.

Publications:

Clark NM, Nothwehr F, Gong M, Maiman LA, Hurwitz ME, Evans D, Roloff DW, Mellins RB. Physician-patient partnership in managing chronic illness. Acad Med 1995;70:957-9.

Contract:

No contracts will be issued for the study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2115-04 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunization Among D.C. Infants

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: B. Simons-Morton Behavioral Scientist PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Howard University, DC General Hospital, Georgetown University Medical Center, Children's Hospital, DC Commission of Public Health, University of DC, Research

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this research is to assess the determinants of non-immunization among DC infants and young children.

Project Description

Objectives: In a sample of low-income, high risk DC mothers of children less than two years of age, identify the determinants of not initiating immunization and failure to maintain immunization status up to date.

Methods Employed:

The research will be conducted over a two-year period, beginning during the 1994-95 fiscal year. 480 mothers will be recruited to an interview after birth in the hospital and at home at 4 and 7 months post-partum.

Significance to Biomedical Research and the Program of the Institute:

Non-immunization during the first two years of life is an important health problem that has been understudied in the U.S.

Proposed Course:

The purpose of this prospective research is to identify predictors of lack of age-appropriate immunization and well child care among D.C. infants and children. At three D.C. hospitals, Howard University Hospital, D.C. General Hospital, and Washington Hospital Center, a cohort of 480 mothers will be recruited to be interviewed in the hospital at birth, and at home or by telephone at 3 months, and 7 months after birth. Assurance of age-appropriate immunization is a national public health priority. Unfortunately, the rates of age appropriate immunizations by age two average about 50% in U.S. cities, and less than 40% in the District of Columbia. Immunization against disease is an important component of pediatric primary health care and serves as an international marker of population health status. Attitudes toward immunization among low income mothers appear to be positive, however, participation in well child care, where most immunization occurs, is inadequate. Only one prospective study has been conducted in the U.S., and the causes of non-participation in well child care and low rates of immunization are largely unknown. The findings from this study will assist policy makers and public health officials in their efforts to increase rates of immunization and improve participation in well child care.

Publications:

None

Contracts:

No contracts are to be issued on this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2116-04 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Preventing Pregnancies Among Adolescents in the District of Columbia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD
Others:	A. Crump	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
	D. Haynie	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Howard University Medical Center, Georgetown University Medical Center, Children's Hospital, DC Commission of Public Health, DC General Hospital

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.45	0.45	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ © Neither

☒ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this research is to test the efficacy of an intervention to prevent adolescent pregnancies. In concert with this effort, a preliminary study involving the collection and analysis of focus group data was conducted to 1) develop an understanding of the perspectives, experiences, and motivations of Washington, D.C. youth with regard to sex, contraception, and pregnancy 2) obtain ideas for and reactions to intervention methods and content and 3) determine the extent to which existing theoretical and programmatic knowledge of teen pregnancy will be applicable to teens in Washington, D.C.

Project Description

Objectives:

In a sample of high risk DC adolescents:

1. Delay the time of initiation of sexual intercourse;

2. Prevent/delay initial and subsequent unintended pregnancies.

Methods Employed:

Subjects will be recruited from public schools in the District of Columbia and randomized to special intervention or usual care conditions. Interventions include an educational curriculum and adjunct, school-based activities. Measures include sexual behavior, contraceptive use, pregnancy status, STDS, and other measures. The research is being conducted over a two year period, which began September 1995.

Major Findings:

Findings are available from the preliminary study involving focus group research with African-American females and males. Two additional focus groups were conducted this year with nulliparous, adolescent, female clinic attenders for a total of 6 focus groups (n=37). In addition, a total of 5 focus groups (n=54) were conducted with adolescent males recruited from a youth employment program.

Adolescent females discussed various factors influencing sexual behavior among their peers. Male influence was manifest in both verbal persuasion from male partners and from the perceived need to engage in sex in order to sustain relationships. Females also described a self-imposed pressure to be like other sexually active teenagers, which may be related to suggestions that sexual activity is a sign of maturity to one's peers. The data suggest that some adolescent females engage in sex for physical pleasure. However, reports were consistent that material gifts are a benefit of sexual relationships for some, although it is less clear whether such gifts are offered for the relationship rather than specifically for sexual intercourse. Females remarked that having sex against one's will is a problem, but descriptions of forcible sex range from blatant coercion to more subtle forms of "taking it." Participants suggested that staying in control in relationships is a matter of respect or how one carries oneself.

Pregnancy is acknowledged as common among peers. However, participants felt that getting pregnant interferes with one's life plans, and comments suggested a strong desire to avoid pregnancy among participants. The only noted advantage of early pregnancy endorsed by some participants was the ability to "grow up with your child." Still participants stated that becoming pregnant intentionally was relatively common among their peers. Reasons for intended pregnancy typically included to gain an attachment to a boy, to have someone to love, and because it's fashionable. Females submit that a substantial number of their parenting peers do not get pregnant intentionally, and for those girls, contraceptive failure, fear of contraceptive side effects, a willingness to take the risk, and simply not thinking about consequences were reasons that pregnancies occurred.

Male focus groups data suggest that peer influence imparted a need to project the image of sexual involvement. Having "main" and "side" partners was not unusual for males, and fidelity seemed to be the exception for their "main" partners. While males described ways that they try to influence girls to engage in sex through persuasion, they also noted that they find some girls to be sexually aggressive as well. Males talked candidly about paternity issues, explaining that it is very important to take care of what you make, although many of their peers don't live up to this standard. Furthermore, paternity issues are complicated by their concerns about whether girls are truthful about paternity. Males suggest that most of their peers don't want their girlfriends to have babies, and typically when pregnancies occur, the male simply was not thinking about the possibility of pregnancy.

Significance to Biomedical Research and the Program of the Institute:

Adolescent pregnancy is an important health problem in need of research. A culturally sensitive intervention to prevent unintended adolescent pregnancies was implemented and the efficiancy of this program is being tested.

Proposed Course:

Preliminary work has been completed and manuscripts are in preparation. The intervention study has begun and will be completed by September 1997.

Publications:

None

Contracts:

No contract is to be issued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2119-03 PRB

PERIOD COVERED

October 1, 1995 through December 31, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Parenting Style and Adolescent Substance Use and Depressive Symptoms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: B. Radziszewska IRTA Post Doctoral Fellow PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

Department of Preventive Medicine, School of Medicine, University of Southern California (J.L. Richardson, C.W. Dent) and School of Public Health, University of Illinois at Chicago (B.R. Flay)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS: 0.15	PROFESSIONAL: 0.15	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research investigates the role of parental supervision and monitoring, parenting style, and parent-adolescent conflict in adolescent substance use and depressive symptoms.

THIS PROJECT HAS BEEN COMPLETED, PLEASE DISCONTINUE

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The goal of this research is to investigate the relationship between parenting styles and practices, parent-adolescent conflict, and adolescent substance use and emotional problems. Two areas are of particular interest: a) The importance of parental supervision and monitoring in the prevention of adolescent problem behavior and adjustment problems, and b) The nature of the relationship between parent-adolescent conflict and adolescent problem behavior and depression.

Methods Employed:

The data were gathered in February 1988, as a part of a larger survey study of adolescent smoking and drug use. The sample consisted of 3993 ninth-grade students in Los Angeles County and San Diego County, representing public schools in six school districts. Trained data collectors provided instructions and administered the questionnaires, which took approximately 45 minutes to complete, to entire classes of students. Letters describing the study were sent to the parents of children in these classrooms.

Significance to Biomedical Research and the Program of the Institute:

Research on the effects of parenting practices has found that certain parenting styles constitute a risk factor for adolescent behavioral and emotional problems, including substance use and depressive symptoms. One parenting style, called "authoritative," which combines parental acceptance with firm behavioral control, is considered a protective factor against negative influences on an adolescent. Research in this area can be useful in formulating new intervention and preventive strategies.

Proposed Course:

Completed.

Publications:

Radziszewska B, Richardson JL, Dent CW, Flay BR. Parenting style and adolescent depressive symptoms, smoking, and academic achievement; ethnic, SES, and gender differences. J Behav Med 1996;19:3; 289-305.

Contract Report:

No contracts were employed for this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2120-02 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Survey of Parent Intervention Strategies to Prevent Adolescent Alcohol Use

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD
OTHERS:	D. Haynie	IRTA Post-Doctoral Fellow	PRB, DESPR, NICHD
	A. Crump	IRTA Post-Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

University of Maryland, Department of Health Education, University of Maryland, College Park (K. Beck)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.55	.55	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research investigates the associations between adolescent alcohol use and parental monitoring practices. Telephone interviews were conducted with randomly selected adolescents (ages 14-19) and their parents in Maryland. Parent monitoring behaviors, parents' perceptions of their adolescents' drinking behavior and adolescents' self reports of drinking behaviors were assessed. Drinking behaviors include underage consumption of alcohol, drinking and driving and riding with a driver who has been drinking. Analyses of these data have revealed that although parents enjoy a variety of strategies to monitor their adolescent's behavior, they significantly underestimate their adolescent's drinking.

Project Description

Objectives:

Parental monitoring practices are thought to have an influence on the likelihood of adolescents engaging in problem behavior. The current study will investigate the associations between parental monitoring and adolescent alcohol consumption, drinking and driving, and riding with an intoxicated driver. Questions of particular interest include: 1) the concordance between parent and adolescent reports of parents' monitoring practices as related to adolescent behavior, 2) the relationship between parent monitoring and enforcement of rules regarding alcohol use and adolescents' self reports of use, 3) parents awareness of their adolescents drinking in relationship to their monitoring behaviors, and 4) age and/or gender differences in parenting practices regarding adolescent drinking.

Methods Employed:

A random telephone survey was conducted with 457 parents and their adolescents in the state of Maryland.

Significance to Biomedical Research and the Program of the Institute:

Underage drinking and driving and driving pose significant risks to adolescent health and well being. Parental monitoring is one of many factors that is important for understanding and addressing this significant health problem. We seek to determine not only what parents say they do to monitor their adolescent children's behavior regarding drinking, but also to assess the extent of concordance between what parents say they do and adolescents report of their parents' monitoring behaviors and expectations for them regarding alcohol. Understanding current parenting practices in relation to adolescent behavior is an important step in designing effective interventions to address underage drinking and adolescent drinking and driving.

Proposed Course:

Data collection was completed in January, 1996 with most of the data entry complete by February, 1996. Data entry of a subset of coded data will be completed by August, 1996. Data analyses began in March, 1996 and will continue through December, 1996. Manuscripts are in preparation and should be ready for submission in December, 1996.

Publications:

None

Contract Report:

No contract issued on this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2121-02 PRB

PERIOD COVERED

October 1, 1995 through September 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Parent-Child Interaction and Asthma Self-Management

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Maiman	Chief	PRB, DESPR, NICHD
Others:	B. Radziszewska	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Public Health, University of Michigan (N.M. Clark); Department of Pediatrics, College of Physicians and Surgeons, Columbia University (D. Evans)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.90	0.90	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of the study is to determine if there is a relationship between the process of parent-child interaction regarding medically recommended self-management behaviors for asthma and the adoption of these behaviors by young adolescents, and, if the adoption of these behaviors influences the child's health status and health care utilization. The survey will be conducted as a "substudy" to an on-going investigation ("main study") of the effects of a physician training program on physician activity related to pediatric asthma-related patient education and adoption of self-management strategies. The subjects in the study are 293 asthmatic children, ages 8-13, and their parents, from two cities: Ann Arbor and New York.

PHS 6040 (Rev. 5/92)

Project Description

Objectives:

The goal of the proposed study is to determine if parent-child interaction regarding asthma self-management influences the adoption of self-management behaviors by young adolescents with asthma, and, if the adoption of these behaviors influences their health status and health care utilization. The specific aims are to examine: 1) the process of collaboration between parents and young adolescents in solving asthma self-management problems; 2) parental behaviors for structuring and managing these interactions; 3) the processes involved in the transfer of responsibility from the parent to the child to manage asthma; 4) young adolescent's information-processing to adopt and follow self-management strategies; 5) geographical and ethnic differences in parent-child communication regarding asthma self-management; and 6) potential systems for delivery of health promotion education to parents and children.

Methods Employed:

The substudy survey will be an adjunct to the main study. Phase I, a unique data collection effort for the substudy, employed face-to-face exploratory interviews and focus groups with both parents and children (N = 32 parent-child pairs) to provide information for the construction of items to be incorporated in the Phase II interviews. Phase II data collection (N = 293 parent-child pairs) will be aligned with the final follow-up telephone interviews of the main study. For parents, the substudy questions will be added to the parent interview. Data collection from children will be a unique effort for the substudy.

Significance to Biomedical Research and the Program of the Institute:

Information from this substudy will be used to help guide future efforts to educate both families and physicians in an attempt to improve the self-management process of parents and children in pediatric asthma and other pediatric conditions, increase the adoption of self-management behaviors by children, and improve child health status and reduce health care utilization.

Proposed Course:

Study design and interview guide construction were developed during October 1995 through February 1996. Phase I (focus interviews) was conducted from March 1996 through July 1996. Data collected at focus interviews will be coded and analyzed from July 1996 through December 1996 and manuscripts prepared. Phase II (final telephone interviews) will be conducted from September 1996 through December 1996 and data will be analyzed.

Publications: None to date.

Contract Report:

No contracts issued on this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2122-02 PRB

PERIOD COVERED

October 1, 1995 to September 30 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Perceptions of Parents Regarding the Onset of Sexual Intercourse Among Youth

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Crump	IRTA Post Doctoral Fellow	PRB, DESPR, NICHD
Others:	L. Maiman	Chief	PRB, DESPR, NICHD
	B. Simons-Morton	Behavioral Scientist	PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

School of Public Health, The Johns Hopkins University (C. Alexander)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.40	0.40	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ © Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The proposed study is a qualitative assessment of the perceptions and behaviors of parents of high risk youth in regard to the prevention of early initiation of intercourse and the negative outcomes associated with this behavior. This research also addresses the strategies parents use to influence their child's behavior and the barriers to implementing these strategies.

Project Description

Objectives:

Early adolescents who are sexually active are at risk of adverse outcomes associated with sexual intercourse. Compared to older youth, early adolescents have less access to information and services needed to prevent pregnancy and sexually transmitted diseases (STDs). In addition, younger teens are still developing cognitive skills that are needed to successfully utilize methods of pregnancy and STD prevention correctly and consistently. This research aims to explore parents' perceptions regarding the problems associated with sexuality among early adolescents and parents' ability to influence this behavior. The objectives of this research are as follows: 1) identify how parents perceive early onset of sexual activity and its potential outcomes 2) identify strategies parents utilize to control or influence sexuality related behaviors of their early adolescents, and 3) identify psychosocial and other barriers to parents' implementation of prevention strategies to control or influence sexuality related behaviors of their early adolescents.

Methods Employed:

Thirty parents of 13-14 year old females participated in the study. These parents were recruited through schools and community organizations to participate in an in-depth interview based on a semi-structured guide. The interviews will be tape recorded and transcribed. Data will be coded and analyzed, and this process will be facilitated by qualitative data analysis software.

Significance to Biomedical Research and the Program of the Institute:

Twenty-three percent of U.S. adolescents have initiated sex by age fourteen, and in populations at high risk for early, unintended pregnancy, as many as half have initiated intercourse at this age. While most interventions to prevent the undesirable outcomes of sexual behavior focus on the adolescent, some recent interventions have attempted to involve parents. Yet little is understood about parents' perceptions of this behavior nor methods parents may utilize to assist their children to prevent adverse outcomes. Understanding parents' perceptions concerning early initiation of sex, STDs and pregnancy is critical to successfully involving them in preventive interventions. This research will provide qualitative information that is needed to generate hypotheses for a quantitative study of parents' perceptions and behaviors.

Proposed Course:

Proposal development began on June 1, 1995, and study design and interview construction began September 1, 1995. The data collection began February 1996 and will be completed by October 1996. Analysis of the data will be completed by December 1996.

Publications:

None

Contract Report:

No contracts issued on this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 2123-01 PRB

PERIOD COVERED

April 1, 1996 through June 30, 1996

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Parental Guidance in Pediatric Asthma Self-Management in Inner-City Population

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Barbara Radziszewska IRTA Post Doctoral Fellow PRB, DESPR, NICHD

COOPERATING UNITS *(if any)*

The Division of Pulmonary and Critical Care Medicine, The Johns Hopkins Hospital, The Johns Hopkins University (C. Rand)

LAB/BRANCH

Prevention Research Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.1	.10	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ © Neither
  ☒ (a1) Minors
  ☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of the study is to determine if there is a relationship between parental guidance related to implementing medically recommended self-management behaviors for asthma and the adoption of these behaviors by children in urban minority populations, and, if the adoption of these behaviors influences the child's health status and health care utilization. The sample in the study consists of 390 children in grades K-5 with moderate to severe asthma, living in Baltimore.

THIS PROJECT IS TERMINATED, PLEASE DISCONTINUE

PHS 6040 (Rev. 5/92)

Project Description

The goal of this study is to examine the relationship between parental guidance behaviors and asthmatic children's adherence to physician recommended medicine regimen and assumption of responsibility for asthma self-management, in an urban minority population. The study focuses on parental approaches used to structure and guide child's asthma self-management activities, and on culture-specific norms and developmental expectations related to children's assumption of responsibility and independence.

Methods Employed:

The "Community Interventions for Minority Children with Asthma" involves telephone interviews with primary caretakers of asthmatic children enrolled in the study.

Significance to Biomedical Research and the Program of the Institute:

Information from this study will be used in developing future asthma education programs for parents and children from urban minority populations. Such programs are urgent, as urban minority children are at increased risk for asthma morbidity and mortality.

Proposed Course:

Questionnaire items covering the above topics were in the process of development, with the intent to add them to the baseline questionnaire in a study conducted by Dr. Rand, titled "Community Interventions for Minority Children with Asthma". Project is terminated due to insufficient resources.

Publications

None

Contract Report:

No contracts were employed for this project.

1949 8854 1
06/04/97 MAB

LIBRARY
NIH
Amazing Research.
Amazing Help.

http://nihlibrary.nih.gov

10 Center Drive
Bethesda, MD 20892-1150
301-496-1080

NIH LIBRARY
3 1496 00719 5509